Iron Biofortification of Staple Crops: Lessons and Challenges in Plant Genetics

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Plants are the ultimate source of iron in our diet, either directly as staple crops and vegetables or indirectly via animal fodder. Increasing the iron concentration of edible parts of plants, known as biofortification, is seen as a sustainable approach to alleviate iron deficiency which is a major global health issue. Advances in sequencing and gene technology are accelerating both forward and reverse genetic approaches. In this review, we summarize recent progress in iron biofortification using conventional plant breeding or transgenics. Interestingly, some of the gene targets already used for transgenic approaches are also identified as genetic factors for high iron in genome-wide association studies. Several quantitative trait loci and transgenes increase both iron and zinc, due to overlap in transporters and chelators for these two mineral micronutrients. Research efforts are predominantly aimed at increasing the total concentration of iron but enhancing its bioavailability is also addressed. In particular, increased biosynthesis of the metal chelator nicotianamine increases iron and zinc levels and improves bioavailability. The achievements to date are very promising in being able to provide sufficient iron in diets with less reliance on meat to feed a growing world population.

Keywords: Crop • Mineral • Nutrition • Phytate.

Introduction

Biofortification of staple crops is widely considered a sustainable and long-term approach to ameliorate nutrient deficiencies. In particular, iron, zinc, selenium and vitamin A are the focus of biofortification programs around the globe, with the aim of complementing and in some cases replacing chemical fortification or food supplementation. Iron deficiency is the most prevalent and widespread nutrient deficiency (WHO 2017). It is the main cause of anemia (~50% of cases), and associated with poor pregnancy outcome, impaired cognitive development, lower immunity and reduced work productivity resulting from tiredness (WHO 2017). While other factors can indirectly lead to iron deficiency, the main cause is low iron intake from diets consisting predominantly of starch-rich, but nutrient poor, staple crops such as white rice, corn meal, wheat flour, potatoes or cassava. While wholegrain cereals have a similar iron concentration to meat products (Table 1), the iron is primarily found in the aleurone and embryo (Fig. 1) and these tissues are removed by postharvest processing (polishing rice and milling of white flours). Moreover, the bioavailability of mineral nutrients in plant foods is significantly lower than in meat because of the presence of antinutrients such as polyphenols and phytic acid in plants (Hurrell and Egli 2010).

The daily requirement for iron (median, absolute values) is 0.71 mg/day for children (7–10 years old), 1.05 mg/day for adult men and 1.46 mg/day for adult women (FAO/WHO 2004). Based on these values, as well as the per capita consumption of staple crops, mineral loss during food preparation and estimated bioavailability, the desired iron concentration for specific staple crops has been calculated (Bouis et al. 2011; Table 1). For crops such as pearl millet and common bean, values close to these targets have been achieved by breeding programs, and there is good evidence from nutrition intervention studies that iron-biofortified crops can indeed increase iron status of target groups (reviewed in Lockyer et al. 2018). However, there is too little genetic variation in iron concentration in the endosperm of cereal grains (especially rice, wheat and corn), therefore transgenic approaches may be the only possible way of obtaining varieties with increased iron.

Research into mineral biofortification has accelerated with the availability of genome sequences, enabling a full experimental cycle of forward and reverse genetics approaches (Fig. 2). Advances in genome-sequencing technology have made it much less time-consuming and cheaper to zoom in on genes underlying quantitative trait loci (QTL), or to identify these genes directly using genome-wide association studies (GWAS). At the same time, >10 years of transgenics studies, using iron homeostasis genes from model organisms and altering their expression in staple crops, have provided invaluable insight into strategies for biofortification.

Increasing Iron by Conventional Breeding

Food crops have been selectively bred for centuries to have desirable traits. The amount of available genetic diversity for a particular trait, which can be exploited by breeding, varies depending on the crop (see Table 1 for iron). The genetics underlying some traits, such as pro-vitamin A accumulation, are well understood and have led to the breeding of crops such
as maize rich in pro-vitamin A (Gebremeskel et al. 2018). For many other traits, including high iron, progress has been slower. Iron homeostasis in plants is tightly regulated, and biofortification often requires circumventing these regulatory mechanisms to allow iron accumulation in a target tissue (Connorton et al. 2017a). While transgenic strategies (see below) target specific genes known to play a role in iron homeostasis, traditional breeding relies on inheritance of the high-iron phenotype, together with a particular genetic marker (Rommens 2007).

There is often less genetic diversity in modern cultivars of most crops compared with older landraces and wild ancestors (Esquinas-Alcázar 2005). For example in wheat, the iron levels of modern cultivars are relatively low and the downward trend in iron concentration is continuing as yield increases (Fan et al. 2008). There is also evidence that environmental factors, such as increasing atmospheric CO2 are likely to lead to a further decline in iron in wheat (Myers et al. 2014). While traditional varieties of rice contain higher concentrations of iron than modern varieties, this may be associated with a yield penalty (Anandan et al. 2011). Nevertheless, there is currently much interest in introducing desirable traits through the introgression of alleles from wild ancestors to modern crops (Palmgren et al. 2015). This has been successful in improving the concentration of iron in grains, e.g. the introgression of chromosomal regions from wild ancestor Aegilops species into modern wheat has led to more than doubling of grain iron (Tiwari et al. 2010, Neelam et al. 2011).

GWAS and QTL mapping have been extremely useful in identifying chromosomal regions, and even specific alleles, associated with high iron in crops. GWAS is able to assess the effect of many different single nucleotide polymorphisms (SNPs) in an unrelated population (Mitchell-Olds 2010). It is useful in determining the effect of polymorphisms in a specific gene, and can help in identifying individual genes playing a role in increasing iron levels. QTL mapping, however, takes advantage of large mapping populations available in many crops, including rice and wheat, and is a powerful tool for studying polygenic, quantitative traits (Collard et al. 2005). Due to the relative scarcity of genetic markers in the genome, QTL often correspond to large chromosomal regions, with sometimes over a hundred genes. Refining the region and identifying particular genes involved can be a challenging next step, and a combination of QTL mapping and GWAS aids this process. Furthermore, as the effect of a particular QTL can be modulated by environmental factors and vary dramatically between studies (Garcia-Oliveira et al. 2018), meta-QTL analysis is a useful tool for combining and integrating information from different studies and can shed light on the genetic architecture behind a particular trait (Wu and Hu 2012). In common bean (Phaseolus vulgaris), a meta-QTL analysis revealed two QTL associated with high iron, and a further eight QTL associated with combined high iron and zinc, across seven studies in South and Middle America (Izquierdo et al. 2018). A total of 12 candidate genes were identified in these QTL, belonging to families including metal transporters (NRAMP, MATE and ZIP), ferric chelate reductases and a bZIP transcription factor (Izquierdo et al. 2018).

| Plant food          | Typical iron concentration (μg/g)a | Natural variation in iron concentration (mg/g DW) (n lines) | Biofortification target set by HarvestPlus (μg/g DW)b | Fold increase |
|---------------------|-----------------------------------|-----------------------------------------------------------|-----------------------------------------------------|---------------|
| Rice, brown         | 15                                | 1–26.8 (274)c                                             | 15                                                  | 7.5×          |
| Rice, polished      | 2                                 | 4–30 (285)c                                              | 59                                                  | 2×            |
| Wheat, wholemeal    | 30                                 | 26.3–68.8 (600)c                                         | 107                                                 | 2.1×          |
| Wheat flour, white  | 7                                 | 5.5–15.7 (43)c                                           | 60                                                  | 2×            |
| Maize, whole        | 30                                 | 11.3–60 (30)c                                            | 107                                                 | 2.1×          |
| Common bean         | 50                                 | 35–93 (1072)d                                           | 88                                                  | 1.9×          |
| Peas, dried         | 50                                 | 23–105 (481)d                                           | 88                                                  | 1.9×          |
| Pearl millet        | 47                                 | 19.7–86.4 (225)c                                        | 88                                                  | 1.9×          |
| Cassava root        | 5                                  | 6–230 (600)c                                            | 45                                                  | 9×            |
| Sweet potato        | 6                                  | 3.2–16.0 (12)c                                          | 85                                                  | 14.2×         |
| Irish potato        | 3                                  | 30–156 (74)c                                            | 88                                                  | 1.9×          |
| Cabbage, broccoli   | 17                                 |                                                            |                                                     |               |
| Tomatoes            | 5                                  |                                                            |                                                     |               |
| Beef steak          | 35                                 |                                                            |                                                     |               |

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aAll values are per gram of the purchased products, which is ‘wet weight’ for cassava, potatoes and vegetables. The iron concentrations in this column can be lower than the values for natural variation in the next column, which are always reported per gram dry weight (FSA 2002).
bBouis et al. (2011).
cGoudia and Hash (2015).
dWhite and Broadley (2009).
eTang et al. (2008).
fLaurie et al. (2012).
gde Haan et al. (2012).
Fig. 1 Physiological processes of iron homeostasis and genes used for iron biofortification. Cross sections of a wheat grain (Triticum aestivum) and common bean seed (P. vulgaris) are shown after staining for iron with Perls’ staining (blue) to show the fundamentally different distribution of iron in these two seed types. Al, aleurone; Em, Embryo; ES, endosperm; Sc, Scutellum; Cot, cotyledon; Plu, plumule with first true leaves; Rad, radical. Scale bar is 1 mm.

Fig. 2 Different research strands enabling iron biofortification of crops. With the revolution in genome sequencing, forward genetics approaches such as QTL mapping and GWAS facilitate the discovery of genes involved in iron homeostasis. Whether polymorphisms in ‘candidate genes’ are the cause of higher iron levels can be verified by using TILLING mutants or gene editing. At the same time, genetic markers in high-iron loci can be used for breeding purposes. In reverse genetics approaches, the expression of known iron homeostasis genes is manipulated to increase the iron concentration of seeds.
Increasing iron uptake

The uptake of iron involves a plethora of genes (Brumbarova et al. 2015, Connorton et al., 2017a) and only a handful of them have thus far been explored as biofortification targets. Plants have two main strategies for iron uptake, the chelate-based strategy in grasses and reductive strategy in non-grasses. Overexpression of IRT1, a divalent metal transporter central to reductive iron uptake, increased the iron concentration in rice leaves by 1.7-fold but only by 1.1-fold in grains (Lee and An 2009). These findings suggest that in the absence of extra sink capacity in the seeds, iron accumulates in the vegetative tissues. Indeed, when IRT1 is overexpressed together with PvFER1 in the endosperm, the iron concentration increased up to 4-fold in polished rice (Boonyaves et al. 2017). In cassava, the combined overexpression of Arabidopsis IRT1 and FER1 resulted in 5.5-fold more iron in tubers (Narayanan et al. 2019). The reductive iron uptake mechanism also involves secretion of small molecules, such as coumarin-derivatives in some species and flavins in others (Connorton et al. 2017a), but whether the biosynthesis genes can be used for biofortification has not been investigated to date. In grasses and cereals, derivatives of NA—mugineic acid and deoxymugineic acid (DMA)—are secreted in the rhizosphere where they chelate Fe$^{3+}$. The Fe-chelator complexes are transported into the cell by YS1 in maize and YSL15 in rice (Curie et al. 2001, Curie et al. 2009). However, increasing DMA by overexpressing ID5S (Iron Deficiency Specific Clone 3, encoding 2’-deoxymugineic-acid 2’-dioxygenase) or overexpressing YSL15 gave only modest increases in the iron concentration in rice seeds (Masuda et al. 2008, Lee et al. 2009a).

Facilitating iron distribution

Iron is transported around the plant in a chelated form, mainly citrate and malate in the xylem, and NA and its derivatives in the phloem. The Fe-NA complexes are transported across membranes by YSL transporters, such as YSL2 in rice (Ishimaru et al. 2010). Biofortification attempts have focused on NA, as it is specifically involved in the transport of divalent metals and not, like citrate, a more general metabolite. Also, the biosynthesis of NA requires only one enzymatic step, mediated by nicotianamine synthase (NAS), which uses S-adenosyl methionine as a substrate. The NAS genes have been overexpressed using strong constitutive promoters such as the Cauliflower Mosaic Virus 35S and ZmUBIQUITIN promoters. This can increase NA levels more than 10-fold in leaves, or even higher in seeds, pushing up iron concentrations approximately 2-fold in grains of rice and wheat (Table 2). In grasses, NA is converted to DMA by nicotianamine aminotransferase.
| Promotera | Genea | Crop/variety | Fold change in transcript or metaboliteb | Fold increase in iron (tissue) | References |
|-----------|-------|-------------|---------------------------------|-------------------------------|------------|
| **Transporters and reductases for Fe uptake** | | | | | |
| ZmUBQ    | OsIRT1 | Rice (Japonica cv. Dongjin) | 10 × (roots and shoots +Fe) | 1.1 (brown seed) | Lee and An (2009) |
| OsACT1   | OsYS1.15 | Rice (Japonica cv. Dongjin) | >100 × (leaves) | 1.3 (brown seed) | Lee et al. (2009a) |
| **Biosynthesis of organic Fe chelators and their transport** | | | | | |
| CaMV 35S | OsNAS1 | Rice (Japonica cv. Nipponbare) | 6 × NA (seed) | 2 (brown seed) | Johnson et al. (2011) |
| CaMV 35S | OsNAS2 | Rice (Japonica cv. Nipponbare) | 9 × NA (seed) | 3 (brown seed) | Johnson et al. (2011) |
| CaMV 35S | OsNAS3 | Rice (Japonica cv. Nipponbare) | 11 × NA (seed) | 4 (polished) 2 (brown seed) | Johnson et al. (2011) |
| CaMV 35S | OsNAS3 | Rice (Japonica cv. Dongjin) | 10 × NA (seed) | 2 (brown seed) | Lee et al. (2009b) |
| ZmUBQ | OsNAS2 | Wheat cv. Bobwhite | 3 × NA (grain) | 1.3 (brown seed) | Beasley et al. (2019) |
| OsACTIN1 | HvNAS1 | Rice (Japonica cv. Tsukinohikari) | 15 × NA (shoots) | 2.5 (polished) | Masuda et al. (2009) |
| CaMV 35S | HvNAS1 | Soybean | 3 × NA (seeds) | 4 (seeds) | Nozoye et al. (2014) |
| CaMV 35S | HvNAS1 | Sweet potato | 7 × NA (leaves) | 2 (storage roots) | Nozoye et al. (2017) |
| HvIDS3 | HvIDS3 | Rice (Japonica cv. Tsukinohikari) | 1.3 (brown seed) | 1.4 (polished) | Masuda et al. (2008) |
| OsSUT1 | OsYS1.2 | Rice (Japonica cv. Tsukinohikari) | 6 × (ear) | 4 (polished seed) | Ishimaru et al. (2010) |
| **Regulators** | | | | | |
| CaMV 35S | OsIRO2 | Rice (Japonica cv. Tsukinohikari) | >60 × (seedlings) | 3 (brown seed) | Ogo et al. (2011) |
| T-DNA insertion | OsHRZ1 | Rice (Japonica cv. Dongjing) | 0.7 × (roots) | 1.7 (brown seed) | Kobayashi et al. (2013) |
| RNAi line | OsHRZ2 | Rice (Japonica cv. Tsukinohikari) | 0.6 × (roots) | 3.5 (brown seed); 3 (polished seed) | Kobayashi et al. (2013) |
| **Fe storage** | | | | | |
| OsGLUB1 | GmFERH1 | Rice (Japonica cv. Kitaake; Indica cv. IR68144) | 3 (brown seed) | 2 (polished) | Goto et al. (1999) and Oliva et al. (2014) |
| OsGLUB1, OsGLUB1 | GmFERH1 | Rice (Japonica cv. Kitaake) | 1.3 (brown) | | Qu et al. (2005) |
| OsGLUA2 | OsFER2 | Rice (Indica cv. Pusa-Sugandh II) | 4.1–7.8 × (polished seed) | 2.1 (polished) | Paul et al. (2012) |
| Zm27gZEIN | GmFERH1 | Maize (B73) | 10 × GmFERH1 vs. native FER | 1.2 (seed) | Kanobe et al. (2013) |
| OsGLUB | PwFER1 | Wheat cv. Bobwhite | 1.6 (grain) | | Singh et al. (2017b) |
| TaGLU-1D-1 | TaFER1-A | Wheat cv. Bobwhite | 50–200 × (endosperm) | 1–1.5 (grain) | Borg et al. (2012) and Neal et al. (2013) |
| TaGLU-1D-1 | TaVIT2-D | Wheat cv. Fielder | 10 × (grain) | 2 (white flour) | Connorton et al. (2017b) |
| TaGLU-1D-1 | OsVIT1 or OsVIT2 | Rice (Japonica cv. Dongjin) | <0.2 × OsVIT1 or OsVIT2 | 1.4 (brown seed) | Zhang et al. (2012) |

(continued)
In plants, iron is stored in the form of ferritin or in vacuoles. Plant ferritin genes have been overexpressed in rice, wheat and maize, using endosperm-specific promoters (Table 2). Expression of the soybean FERH1 gene in rice led to a 3-fold increase in iron concentration in unpolished or polished grains, but the same gene was less effective in maize. In wheat, only a 1.5-fold increase in iron concentration was found by expressing FER1 from common bean (P. vulgaris) or from wheat itself (Borg et al. 2012, Neal et al. 2013, Singh et al. 2017a). Assuming that all constructs are similarly effective in raising ferritin expression levels, it may be that rice is able to direct more iron to ferritin than wheat. Interestingly, overexpression of a vacuolar iron transporter, TaVIT2, with the same endosperm-specific GLU1D-1 promoter used for ferritin overexpression, raised iron levels more than 2-fold (Connorton et al. 2017b). Enhancing vacuolar iron storage may be more effective than ferritin iron storage because the former is how iron is stored normally in cereal grains. Overexpression of VIT genes in rice has not been fully explored, but knockdown or knockout of VIT1 or VIT2 led to iron accumulation in the embryo, and increased the total iron concentration in brown rice by approximately 25–30% or 1.3-fold (Zhang et al. 2012, Bashir et al. 2013a). The iron concentration of roots and shoots of the VIT mutant lines was lower than in wild type. Thus, decreased storage capacity for iron in these parts of the plant seemed to redirect iron to the seed and embryo (but not the endosperm). Overexpression of Arabidopsis VIT1 in the tubers of cassava was also effective, raising iron 3- to 4-fold. Taken together, increasing the storage capacity of iron works well as a single-gene strategy, but whether ferritin or vacuolar storage should be used may differ from species to species.

### Enhancing iron storage

In addition to targeting one aspect of iron homeostasis, multigene approaches to simultaneously increase iron uptake, distribution and storage have been very successful (Table 2). Another way of changing the expression of several genes would...
be to interfere with global regulators of iron homeostasis. For example, overexpression of the transcription factor OsIRO2, which activates a number of genes for iron uptake, resulted in a 3-fold increase in the iron concentration in brown rice (Ogo et al. 2011). Other promising targets are the HRZ1 and HRZ2 genes, which act as negative regulators of the transcriptional response to iron deficiency. Rice mutants in HRZ1 or HRZ2 have constitutively induced transcript levels of genes for iron uptake and mobilization (Kobayashi et al. 2013), and they accumulate 1.7- to 3.5-fold iron in seeds. Although seed viability is diminished in hrz1 mutant lines, perhaps when combined with increased iron storage capacity this could be an efficient biofortification strategy in the future.

**Improving Iron Bioavailability**

Bioavailability broadly refers to the proportion of a nutrient that is absorbed from the diet and used for normal body functions. As already mentioned, the bioavailability of iron is low (<15%) in plant foods, and therefore an important factor to consider in biofortification approaches of staple crops. Bioavailability is not straightforward to measure, and depends not only on external factors such as the food matrix and the chemical form of the nutrient, but also on internal factors including gender, age, nutrient status and life stage (e.g. pregnancy) (Hurrell and Egli 2010). Showing that biofortification of a certain crop leads to better iron nutrition requires human intervention studies which can be demanding logistically and come at considerable costs. As a proxy for bioavailability, the absorption of iron into Caco-2 cells can be measured (Glahn et al. 1998). This is a human cell line that morphologically and functionally resembles the enterocytes lining the small intestine, except that they are not protected by a mucosal layer. Iron absorption is measured by the amount of human ferritin that is formed in response to added iron sulfate (positive control) or a digested food matrix (Glahn et al. 1998). The in vitro Caco-2 cell assays can also reveal the inhibitory effects of antinutrients such as phytic acid. Animal studies of iron bioavailability have been performed using mice or chickens, but these models do not fully recapitulate nutrient absorption in the human gut. The biophysical measure of iron bioaccessibility, which is the potential availability of a mineral in food for absorption in the gut following digestion, may also provide some indication of bioavailability (Narayanan et al. 2019).

While the chemical form (speciation) of iron affects its bioavailability, the speciation of iron in plant foods is dramatically altered by food preparation (cooking) and intestinal digestion. For example, Fe(III)hydroxides in ferritin are released as Fe\(^{2+}\) or Fe\(^{3+}\) during heating (Hoppler et al. 2008), and likely chelated by phytic acid in the food matrix (Moore et al. 2018, Perfecto et al. 2018). Overall, the amount of phytic acid is a strong indicator for bioavailability, and in the absence of any enhancers of iron absorption a molar ratio of phytic acid: iron of <1:1 is required for significantly improved absorption of iron from cereal-based meals (Hurrell and Egli 2010). In some pulses and wholegrain cereals, the molar ratio is \(\geq 10:1\) and bioavailability is effectively zero in Caco-2 cell culture (Rodriguez-Ramiro et al. 2017), but a fractional absorption of 4–6% iron was measured in young women by isotope studies (Petry et al. 2013). Efforts to breed crops with lower phytic acid have been reported, although a strong decrease in phytic acid may affect yield. A 60% decrease in phytic acid in pea was shown to improve iron bioavailability in Caco-2 cell studies (Warkentin et al. 2012, Liu et al. 2015) and common bean carrying the *low phytic acid (lpa)* trait improved the iron status of young females (Petry et al. 2013). Alternatively, phytic acid has been lowered by expressing a fungal phytase resulting in 3-fold more bioavailable iron in maize (Drakakaki et al. 2005). In cereal grains, phytic acid is mostly localized to the bran, in contrast to low levels in the endosperm (Fig. 1). Therefore, targeting iron specifically to the endosperm is an alternative solution to the ‘phytate problem’ (Connorton et al. 2017b).

Differences in iron bioavailability are not exclusively attributable to differences in phytic acid levels. QTL controlling total grain iron barely overlapped with bioavailability QTL and combining three of the larger QTL led to higher iron bioavailability (Lung’aho et al. 2011). In parallel, several independent studies have shown that increasing the NA content, either by overexpressing NAS genes in rice and wheat (Zheng et al. 2010, Beasley et al. 2019) or by adding NA to the food matrix (Zheng et al. 2010), enhances bioavailability of iron in mice or in Caco-2 cell culture. However, human intervention studies have thus far not been conducted.

**Concluding Remarks**

Both conventional breeding and transgenic approaches have shown that it is possible to increase iron concentrations in staple crops. More than 10 years of transgenic approaches have revealed useful lessons for future developments of biofortification. For example, it has taught us that increasing the uptake of iron from the soil needs to be combined with increased iron storage capacity. Whether vacuolar or ferritin iron storage should be targeted depends on the plant species and tissue. Promoters and genes, or gene paralogs, appear to be equally effective in different crop species. Also, it has been observed that some transgenic strategies increase iron specifically, whereas others (e.g. NAS genes) increase both iron and zinc levels. And finally, an important finding is that increased biosynthesis of NA also improves the bioavailability of iron.

 Doubtlessly more gene targets remain to be explored. In particular, rather than overexpressing several transgenes, manipulating a single regulatory gene either by changing expression levels or by modulating its activity, may achieve a similar transcriptional response. Studies on *IRO2* and the *HRZ* genes in rice are the start of this approach, and further gene targets may be identified by forward genetics approaches such as QTL mapping or GWAS. While forward genetic studies in the model crop Arabidopsis could also be useful for gene discovery, this should then focus on iron homeostasis in seeds and not other tissues. In addition, the proteins that mediate iron transfer from the maternal tissues to the embryo are little known,
and their overexpression could greatly enhance iron loading of seeds.

It is important for plant scientists to work together with nutritionists, to test iron bioavailability of biofortified crops in vitro studies or in human intervention studies. Discussions with researchers involved in implementation of high-iron crops may help direct the research toward particular crops or specific plant foods. And ultimately, a broader discussion with the public about ‘nutritious foods’ is required to increase the acceptance of iron-biofortified crop varieties in addition to existing practices of chemical fortification and iron supplements. In particular for rice and wheat, transgene expression or gene editing are necessary to achieve sufficient iron levels in a meat-free diet. Encouragingly, genetically modified crops are already accepted and grown in several countries.

Disclosures

The authors have no conflicts of interest to declare.

References

Alomari, D., Eggett, K., von Wirén, N., Polley, A., Plieske, J., Canal, M., et al. (2018) Whole-genome association mapping and genomic prediction for iron concentration in wheat grains. *Int. J. Mol. Sci.* 20: 76.

Anandan, A., Rajiv, G., Eswaran, R. and Prakash, M. (2011) Genotypic variation and relationships between quality traits and elemental contents in traditional and improved rice (*Oryza sativa* L.) genotypes. *J. Food Sci. Technol.* 56: 233–240.

Aung, M.S., Masuda, H., Kobayashi, T., Nakanishi, H., Yamakawa, T. and Nishizawa, N.K. (2013) Iron biofortification of Myanmar rice. *Front. Plant Sci.* 4: 158.

Banakar, R., Álvarez Fernández, A., Abadía, J., Capell, T. and Christou, P. (2017a) The expression of heterologous Fe (III) phytosiderophore transporter HvSIT2 in rice increases Fe uptake, translocation and seed loading and decreases heavy metals by selective Fe transport. *Plant Biotechnol. J.* 15: 423–432.

Banakar, R., Alvarez Fernandez, A., Diaz-Benito, P., Abadia, J., Capell, T. and Christou, P. (2017b) Phytosiderophores determine thresholds for iron and zinc accumulation in biofortified rice endosperm while inhibiting the accumulation of cadmium. *J. Exp. Bot.* 68: 4983–4995.

Bashir, K., Inoue, H., Nagasaka, S., Takahashi, M., Nakanishi, H., Mori, S. and et al. (2006) Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. *J. Biol. Chem* 281: 32395–32402.

Bashir, K., Takahashi, R., Akhtar, S., Ishimaru, Y., Nakanishi, H. and Nishizawa, N.K. (2013a) The knockdown of OsVIT2 and MFT affects iron localization in rice seed. *Rice (N Y)* 6: 31.

Bashir, K., Takahashi, R., Nakanishi, H. and Nishizawa, N.K. (2013b) The road to micronutrient biofortification of rice: progress and prospects. *Front. Plant Sci.* 4: 15.

Beasley, J.T., Bonneau, J.P., Sánchez-Palacios, J.T., Moreno-Moyano, L.T., Callahan, D.L., Tako, E. and et al. (2019) Metabolic engineering of bread wheat improves grain iron concentration and bioavailability. *Plant Biotechnol. J.* 1–13. doi: 10.1111/pbi.13074.

Beneč, I., Schreiber, K., Ripperger, H. and Kircheiss, A. (1983) Metal complex formation by nicotianamine, a possible phytosiderophore. *Experientia* 39: 261–262.

Boonyaves, K., Gruissem, W. and Bhullar, N.K. (2016) NOD promoter-controlled AtIRT1 expression functions synergistically with NAS and FERRITIN genes to increase iron in rice grains. *Plant Mol. Biol.* 90: 207–215.

Boonyaves, K., Wu, T.-Y., Gruissem, W. and Bhullar, N.K. (2017) Enhanced grain iron levels in rice expressing an IRON-REGULATED METAL TRANSPORTER, NICOTIANAMINE SYNTHASE, and FERRITIN gene cassette. *Front. Plant Sci.* 8: 130.

Borg, S., Brinch-Pedersen, H., Tauris, B., Madsen, L.H., Darbani, B., Noepavar, S. and et al. (2012) Wheat ferritins: improving the iron content of the wheat grain. *J. Cereal Sci.* 56: 204–213.

Bouis, H.E., Horst, C., McClafferty, B., Meenakshi, J.V. and Pfeiffer, W.H. (2011) Biofortification: a new tool to reduce micronutrient malnutrition. *Food Nutr. Bull.* 32: 31–40.

Brumbarova, T., Bauer, P. and Ivanov, R. (2015) Molecular mechanisms governing Arabidopsis iron uptake. *Trends Plant Sci.* 20: 124–133.

Clemens, S., Deinlein, U., Ahmadi, H., Höretz, S. and Uraguchi, S. (2013) Nicotianamine is a major player in plant Zn homeostasis. *Biometals* 26: 623–632.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B. and Pang, E.C.K. (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. *Euphytica* 146: 169–196.

Connorton, J.M., Balk, J. and Rodriguez-Celma, J. (2017a) Iron homeostasis in plants—a brief overview. *Metallomics* 9: 813–823.

Connorton, J.M., Jones, E.R., Rodriguez-Ramiro, I., Fairweather-Tait, S., Uauy, C. and Balk, J. (2017b) wheat vacuolar iron transporter TaVIT2 transports Fe and Mn and is effective for biofortification. *Plant Physiol.* 174: 2434–2444.

Crespo-Herrera, L.A., Govindan, V., Stangoulis, J., Hao, Y. and Singh, R.P. (2017) QTL mapping of grain Zn and Fe concentrations in two hexaploid wheat RIL populations with ample transgressive segregation. *Front. Plant Sci.* 8: 1800.

Crespo-Herrera, L.A., Vela, G. and Singh, R.P. (2016) Quantitative trait loci mapping reveals pleiotropic effect for grain iron and zinc concentrations in wheat. *Ann. Appl. Biol.* 169: 27–35.

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M. and et al. (2009) Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* 103: 1–11.

Curie, C., Panaviene, Z., Lougerue, C., DellaPorta, S.L., Briat, J.F. and Walker, E.L. (2001) Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409: 346–349.

de Haan, S., Burgos, G., Caanto, R., Arcos, J., Scarrrah, M., Salas, E. and et al. (2012) Effect of production environment, genotype and process on the mineral content of native bitter potato cultivars converted into white chuhuio. *J. Sci. Food Agric.* 92: 2098–2105.

Descalsola, G.I.L., Swamy, B.P.M., Zaw, H., Inabangan-Asilo, M.A., Amparado, A., Mauleon, R. and et al. (2018) Genome-wide association mapping in a rice MAGIC Plus population detects QTLs and genes useful for biofortification. *Front. Plant Sci.* 9: 1347.

Drakakaki, G., Marcel, S., Glahn, R.P., Lund, E.K., Pariagh, S., Fischer, R. and et al. (2005) Endosperm-specific co-expression of recombinant soybean ferritin and Aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. *Plant Mol. Biol.* 59: 869–880.

Esquinas-Alcázar, J. (2005) Protecting crop genetic diversity for food security: political, ethical and technical challenges. *Nat. Rev. Genet.* 6: 946–953.

Fan, M.S., Zhao, F.J., Fairweather-Tait, S.J., Poulton, P.R., Dunham, S.J. and McGrath, S.P. (2008) Evidence of decreasing mineral density in wheat grain over the last 160 years. *J. Trace Elem. Med. Biol.* 22: 207–215.

Fan, M.S., Zhao, F.J., Fairweather-Tait, S.J., Poulton, P.R., Dunham, S.J. and McGrath, S.P. (2008) Evidence of decreasing mineral density in wheat grain over the last 160 years. *J. Trace Elem. Med. Biol.* 22: 207–215.

FAO/WHO. (2004) Vitamin and Mineral Requirements in Human Nutrition. https://apps.who.int/nutritiondata/handle/10665/42716/9241546123.pdf (February 4, 2019, date last accessed).

FSA. (2002) McCance and Widdowson’s the Composition of Foods, 6th Summary edn. Royal Society of Chemistry, Cambridge.
Garcia-Oliveira, A.L., Chander, S., Ortiz, R., Menkir, A. and Gedil, M. (2018) Genetic basis and breeding perspectives of grain iron and zinc enrichment in cereals. Front. Plant Sci. 9: 937.

Gebremeskil, S., Garcia-Oliveira, A.L., Menkir, A., Adetimirin, V. and Gedil, M. (2018) Effectiveness of predictive markers for marker assisted selection of pro-vitamin A carotenoids in medium-late maturing maize (Zea mays L.) inbred lines. J. Cereal Sci. 79: 27–34.

Glahn, R.P., Lee, O.A., Yeung, A., Goldman, M.I. and Miller, D.D. (1998) Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. J. Nutr. 128: 1555–1561.

Goto, F., Yoshihara, T., Shigemoto, N., Toki, S. and Takaika, F. (1999) Iron fortification of rice seed by the soybean ferritin gene. Nat. Biotechnol. 17: 282–286.

Goudia, B.D. and Hash, C.T. (2015) Breeding for high grain Fe and Zn levels in maize. Int. J. Innov. Appl. Stud. 12: 342–345.

Hoppler, M., Schönbachler, A., Mele, L., Hurrell, R.F. and Walczyk, T. (2008) Ferritin-iron is released during boiling and in vitro gastric digestion. J. Nutr. 138: 878–884.

Hurrell, R. and Egli, I. (2010) Iron bioavailability and dietary reference values. Am. J. Clin. Nutr. 91: 1461S–1467S.

Ishimaru, Y., Masuda, H., Bashi, K., Inoue, H., Tsukamoto, T., Takahashi, M., et al. (2010) Rice metal-nicotianamine transporter, OsYSII2, is required for the long-distance transport of iron and manganese. Plant J. 62: 379–390.

Izquierdo, P., Astudillo, C., Blair, M.W., Iqbal, A.M., Raatz, B. and Cichy, K.A. (2018) Meta-QTL analysis of seed iron and zinc concentration and content in common bean (Phaseolus vulgaris L.). Theor. Appl. Genet. 131: 1645–1658.

Jin, T., Zhou, J., Chen, J., Zha, L., Zhao, Y. and Huang, Y. (2013) The genetic architecture of zinc and iron content in maize grains as revealed by QTL mapping and meta-analysis. Breed. Sci. 63: 317–324.

Johnson, A.A.T., Kyriacou, B., Callahan, D.L., Carruthers, L., Stangoulis, J., Jin, T., Zhou, J., Chen, J., Zhu, L., Zhao, Y. and Huang, Y. (2013) The genetic content in common bean (Phaseolus vulgaris L.). Theor. Appl. Genet. 131: 1645–1658.

Kawakami, Y. and Bhullar, N.K. (2018) Molecular processes in iron and zinc accumulations in rice. Plant Physiol. 174: 75–88.

Kobayashi, T., Nagasaka, S., Senoura, T., Itai, R.N., Nakanishi, H. and Nishizawa, N.K. (2013) Iron-binding haemerythrin RING ubiquitin ligases regulate plant iron responses and accumulation. Nat. Commun. 4: 2792.

Laurie, S.M., van Jaarsveld, P.J., Faber, M., Philpott, M.F. and Labuschagne, M.T. (2012) Changes in endogenous gene transcript and protein levels in maize plants expressing the soybean ferritin transgene. Front. Plant Sci. 4: 196.

Lee, S. and An, G. (2009) Over-expression of OsIRT1 leads to increased iron and zinc accumulations in rice. Plant Cell Environ. 32: 408–416.

Lee, S., Cheicko, J.C., Kim, S.A., Walker, E.L., Lee, Y., Guerinot, M.L., et al. (2009a) Disruption of OsYSI15 leads to iron inefficiency in rice plants. Plant Physiol. 150: 786–800.

Lee, S., Jeon, U.S., Lee, S.J., Kim, Y.-K., Persson, D.P., Husted, S., et al. (2009b) Iron fortification of rice seeds through activation of the nicotianamine synthase gene. Proc. Natl. Acad. Sci. USA 106: 22014–22019.

Liu, X., Glahn, R.P., Arganosa, G.C. and Warkentin, T.D. (2015) Iron bioavailability in low phytate pea. Crop Sci. 55: 320.

Lockyer, S., White, A. and Butteriss, J.L. (2018) Biofortified crops for tackling micronutrient deficiencies—what impact are these having in developing countries and could they be of relevance within Europe? Nutr. Bull. 43: 319–357.

Lung’aho, M.G., Mwaniki, A.M., Szalma, S.J., Hart, J.J., Rutzke, M.A., Kochian, L.V., et al. (2011) Genetic and physiological analysis of iron biofortification in maize kernels. PLoS One 6: 1–10.

Masuda, H., Aung, M.S. and Nishizawa, N.K. (2013a) Iron biofortification of rice using different transgenic approaches. Rice (N Y) 6: 40.

Masuda, H., Ishimaru, Y., Aung, M.S., Kobayashi, T., Kakei, Y., Takahashi, M., et al. (2012) Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. Sci. Rep. 2: 543.

Masuda, H., Kobayashi, T., Ishimaru, Y., Takahashi, M., Aung, M.S., Nakanishi, H., et al. (2013b) Iron-biofortification in rice by the introduction of three barley genes participated in mugineic acid biosynthesis with soybean ferritin gene. Front. Plant Sci. 4: 132.

Mitchell-Olds, T. (2010) Complex-trait analysis in plants. Genome Biol. 11: 113.

Moore, K.L., Rodríguez-Ramiro, I., Jones, E.R., Jones, E.J., Rodríguez-Celma, J., Halsey, K., et al. (2018) The stage of seed development influences iron bioavailability in pea (Pisum sativum L.). Sci. Rep. 8: 6865.

Neelam, K., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A.D.B., Bloom, A.J., et al. (2014) Increasing CO2 threatens human nutrition. Nature 510: 139–142.

Narayan, N., Beyene, G., Chauhan, R.D., Gaitán-Solís, E., Gehan, J., Butts, P., et al. (2019) Biofortification of field-grown cassava by engineering expression of an iron transporter and ferritin. Nat. Biotechnol. 37: 144–151.

Narayan, N., Beyene, G., Chauhan, R.D., Gaitán-Solís, E., Gruskas, M.A., Taylor, N., et al. (2015) Overexpression of Arabidopsis VIT1 increases accumulation of iron in cassava roots and stems. Plant Sci. 240: 170–181.

Neal, A.L., Geraki, K., Borg, S., Quinn, P., Mosselmans, J.F., Brinch-Pedersen, H., et al. (2013) Iron and zinc complexation in wild-type and ferritin-expressing wheat grain: implications for mineral transport into developing grain. J. Biol. Inorg. Chem. 18: 557–570.

Neelam, K., Rawat, N., Tiwari, V.K., Kumar, S., Chhuneja, P., Singh, K., et al. (2011) Introgression of group 4 and 7 chromosomes of Aegilops tauschii into wheat enhances grain iron and zinc density. Mol. Breed. 28: 633–634.

Nozoye, T., Kim, S., Kakei, Y., Takahashi, M., Nakanishi, H. and Nishizawa, N.K. (2014) Enhanced levels of nicotianamine promote iron accumulation and tolerance to calcareous soil in soybean. Biosci. Biotechnol. Biochem. 78: 1677–1684.

Nozoye, T., Otani, M., Senoura, T., Nakanishi, H. and Nishizawa, N.K. (2017) Overexpression of barley nicotianamine synthase 1 confers tolerance in the sweet potato to iron deficiency in calcareous soil. Plant Soil 418: 75–88.

Ogo, Y., Itai, R.N., Kobayashi, T., Aung, M.S., Nakanishi, H. and Nishizawa, N.K. (2011) OsIRO2 is responsible for iron utilization in rice and improves growth and yield in calcareous soil. Plant Mol. Biol. 75: 593–605.

Oliva, N., Chada-Mohanty, P., Poletti, S., Abrigo, E., Atenzia, G., Torizzi, L., et al. (2014) Large-scale production and evaluation of marker-free indica rice IR64 expressing phytoferritin genes. Mol. Breed. 33: 23–37.

Palmgren, M.G., Edenbrandt, A.K., Vedel, S.E., Andersen, M.M., Landes, X., Østerberg, J.T., et al. (2015) Are we ready for back-to-nature crop breeding? Trends Plant Sci. 20: 155–164.

Pau, S., Ali, N., Gayen, D., Datta, S.K. and Datta, K. (2012) Molecular breeding of Osfer2 gene to increase iron nutrition in rice grain. GM Crops Food. 3: 310–316.

Perfecto, A., Rodríguez-Ramiro, I., Rodríguez-Celma, J., Sharp, P., Baker, J. and Fairweather-Tait, S. (2018) Pea ferritin stability under gastric pH
conditions determines the mechanism of iron uptake in Caco-2 cells. J. Nutr. 148: 1229–1235.
Petry, N., Egli, I., Campion, B., Nielsen, E. and Hurrell, R. (2013) Genetic reduction of phytate in common bean (Phaseolus vulgaris L.) seeds increases iron absorption in young women. J. Nutr. 143: 1219–1224.
Qu, L.Q., Yoshihara, T., Ooyama, A., Goto, F. and Takaiwa, F. (2005) Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. Planta 222: 225–233.
Rodriguez-Ramíro, I., Brearley, C.A., Bruggraber, S.F.A., Perfecto, A., Shewry, P. and Fairweather-Tait, S. (2017) Assessment of iron bioavailability from different bread making processes using an in vitro intestinal cell model. Food Chem. 228: 91–98.
Rommens, C.M. (2007) Intragenic crop improvement: combining the benefits of traditional breeding and genetic engineering. J. Agric. Food Chem. 55: 4281–4288.
Singh, S.P., Gruissem, W. and Bhullar, N.K. (2017a) Single genetic locus improvement of iron, zinc and β-carotene content in rice grains. Sci. Rep. 7: 6883.
Singh, S.P., Keller, B., Gruissem, W. and Bhullar, N.K. (2017b) Rice NICOTIANAMINE SYNTHASE 2 expression improves dietary iron and zinc levels in wheat. Theor. Appl. Genet. 130: 283–292.
Tang, J., Zou, C., He, Z., Shi, R., Ortiz-Monasterio, I., Qu, Y., et al. (2008) Mineral element distributions in milling fractions of Chinese wheats. J. Cereal Sci. 48: 821–828.
Tiwari, V.K., Rawat, N., Neelam, K., Kumar, S., Randhawa, G.S. and Dhaliwal, H.S. (2010) Substitutions of 25 and 7U chromosomes of Aegilops kotschyi in wheat enhance grain iron and zinc concentration. Theor. Appl. Genet. 121: 259–269.
Trijatmiko, K.R., Dueñas, C., Tsakirpaloglu, N., Torrilo, L., Arines, F.M., Adeva, C., et al. (2016) Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. Sci. Rep. 6: 19792.
Uauy, C., Distelfeld, A., Fahima, T., Blechl, A. and Dubcovsky, J. (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314: 1298–1301.
Vasconcelos, M.W., Gruissem, W. and Bhullar, N.K. (2017) Iron biofortification in the 21st century: setting realistic targets, overcoming obstacles, and new strategies for healthy nutrition. Curr. Opin. Biotechnol. 44: 8–15.
Vert, G., Grotz, N., Dédalchémond, F., Gaymard, F., Guerinot, M.L., Briat, J.F., et al. (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell. 14: 1223–1233.
Warkentin, T.D., Delgerjav, O., Arganosa, G., Rehman, A.U., Bett, K.E., Anbessa, Y., et al. (2012) Development and characterization of low-phytate pea. Crop Sci. 52: 74–78.
White, P.J. and Broadley, M.R. (2009) Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol. 182: 49–84.
WHO. (2017) Malnutrition. www.who.int/mediacentre/factsheets/malnutrition/en/ (February 4, 2019, date last accessed).
Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., et al. (2009) Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. Plant Biotechnol. J. 7: 631–644.
Wu, X.-L. and Hu, Z.-L. (2012) Meta-analysis of QTL mapping experiments. In Quantitative Trait Loci: Methods and Protocols. Edited by Rifkin, S.A. pp. 145–171. Humana Press, Totowa, NJ.
Zhang, Y., Xu, Y.-H., Yi, H.-Y. and Gong, J.-M. (2012) Vascular membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. Plant J. 72: 400–410.
Zheng, L., Cheng, Z., Ai, C., Jiang, X., Bei, X., Zheng, Y., et al. (2010) Nicotianamine, a novel enhancer of rice iron bioavailability to humans. PLoS One. 5: e10190.