



Biofortification of major crop plants with iron and zinc - achievements and future directions

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Abstract Biofortification is a long-term strategy of delivering more iron (Fe) and zinc (Zn) to those most in need. Plant breeding programs within the CGIAR and NARS have made major advances in Fe- and Zn-dense variety development and there have been successful releases of new biofortified varieties. Recent research effort has led to a substantial improvement in our knowledge of Fe and Zn homeostasis and gene regulation, resulting in the identification of candidate genes for marker assisted selection. International cooperation between the agricultural and nutrition community has been strengthened, with numerous implementation and partnership strategies developed and employed over the years. The evidence on the effectiveness of Fe and Zn biofortified crops is slowly building up and the results are encouraging. Biofortification continues to be scaled out and further work is required to reach the general aim of eradicating the hidden hunger of Fe and Zn deficiency in the world's population and ensuring nutritional security.

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Introduction

The global population is expected to increase from 7.6 billion in 2017 to 8.6 billion in 2030 and 9.8 billion by 2050 (United Nations 2016). Currently, 40% of pregnant women and 42% of children below 5 years of age are anaemic and 17% of the global population is at risk of inadequate Zn intakes (WHO 2021). The task of feeding this growing population with both sufficient food and Fe and Zn, is of critical importance.

Dietary diversity is the optimal route for delivering adequate nutrition but diets of low-income, poor families in developing countries predominantly involve staple based, plant products and many of these foods (such as cereals) are low in nutrients (Bouis and Welch 2010).

With the advent of the Green Revolution, higher-yielding cereal crops were developed and while this was critical for providing more staple food to undernourished populations, it also led to a reliance on low micronutrient staples in the diet (Welch and Graham 2004). A historical example of the effects of selecting on high yield and not considering micronutrients, can be visualised with wheat. Over

a 120-year period of taking records, hard white wheat varieties were shown to have an increase in yield of up to 175% but this coincided with a decrease of 11 to 25% in Fe and Zn concentration, respectively (Murphy et al. 2008). The cause for this is not entirely understood but it is thought to be due to a possible “dilution effect” caused by the increase in grain size and a change in the ratio of bran to endosperm in modern varieties (Murphy et al. 2008). Biofortification aimed to rectify this imbalance by not only breeding for improved agronomic performance, but also redirecting efforts to improve the Fe and Zn concentration of new varieties being developed.

A question often posed is whether we can have both high yield and high micronutrient concentration? In the case of wheat, despite the historically inverse relationship between yield and micronutrient concentrations, it has been shown that these traits are controlled by separate genetic pathways (Velu et al. 2018a), consequently it is feasible to breed for both high yield and high nutrient genotypes, thereby negating the dilution effect due to high yield. Furthermore, (Velu et al. 2018a) have shown that QTL for larger seed size and Zn concentration are co-located, thereby enabling the simultaneous increase of yield and micronutrients using this co-associated, genetic loci. Likewise, the overexpression of HvSUT-1 in wheat led to a significant increase in yield, seed size and an increase in Fe and Zn concentration (Saalbach et al. 2014).

Today, several breeding programs within the CGIAR network have biofortification as a core trait and therefore we are assured of new varieties in these programs, having both good agronomic performance and high nutrient concentration. Today, the current global challenge of Fe and Zn deficiencies in human populations can be prevented with timely and adequately implemented biofortification strategies which tend to reach the most affected population groups; malnourished rural populations with low dietary diversity, limited access to supplementation and fortified foods (Bouis and Welch 2010). Biofortification has shown itself to be a sustainable strategy aimed at increasing the concentrations of Fe and Zn in edible crop plants, reducing the negative consequences of commonly consumed Fe and/or Zn deficient crops, alleviating Zn and Fe deficiency in people, and intended to help in eradicating the hidden hunger

of the growing population by 2030 (United Nations 2016).

With the advent of biofortification came a heightened focus on Fe and Zn homeostasis, to further improve our knowledge of the physiology of these two elements and the genetics related to improving Fe and Zn nutrition of the edible portion of crop plants. Inherent variation in Fe and Zn was exploited within germplasm banks, to try and meet Fe and Zn targets developed by the nutrition community.

When screening germplasm, questions arose as to the heritability of the trait, versus the effects of environment and whether the genetics were sufficient to enable a breeding program. Concurrently, plant physiologists needed to better understand the mechanisms associated with the transport of more Fe and Zn into seeds, tubers, and edible roots. Since the advent of biofortification, there have been numerous reports in the literature around these central themes.

The founding fathers of biofortification, Professor Ross Welch, Professor Robin Graham, and Dr. Howarth Bouis grasped the concept of using agriculture to deliver improved nutrition to an estimated 2 billion people worldwide who are suffering from hidden hunger, where one-third of the global population is affected by Zn and Fe deficiency (Beal et al. 2017). Their foresight has led to a global movement to not only see agriculture as a means to provide caloric energy, but also to provide essential micronutrients. Furthermore, we are now thinking more about the bioavailability of Fe and Zn and it not just about their intake. Antinutritional factors and promoters of absorption are on the agenda for genetic manipulation to enhance the absorption of Fe and Zn in the human gut.

This review summarises and discusses major challenges, achievements, and benefits gained through Zn and Fe biofortification of crop plants made in the last twenty-five years, with more of an emphasis on the cereal crops. It describes the most important approaches used to accomplish Zn and Fe biofortification goals and discusses promising strategies for effective crop biofortification in the future. Further work is required to ensure that multi-micromineral, nutritionally adequate crops are available in adequate amounts across the globe. While we acknowledge that Vitamin A is also a major deficiency among many in the developing world, this review will focus on Fe and Zn.

Biofortification of staple food crops

The two streams to achieve a biofortified crop are conventional plant breeding and fertiliser agronomy and these complementary routes have been a major accomplishment since the advent of the biofortification strategy (Cakmak 2008; Knez et al. 2018; Saha et al. 2017; Prom-U-Thai et al. 2020; Zou et al. 2019).

Plant breeding

HarvestPlus was borne out of a CGIAR Challenge Program and has a vision of a world free of deficiencies such as Fe, Zn and Vitamin A (for a detailed vision, mission and goal statement, see www.harvestplus.org/about/our-mission). HarvestPlus has led the way in terms of the plant breeding strategy, firstly to develop target concentrations across a broad range of crops and then to initiate a breeding approach of first assessing whether stable genetic variability exists for Fe and Zn within a crop, and this involved screening germplasm, varieties and elite breeding lines (Andersson et al. 2017). The heritability of micronutrient concentrations in edible plant part was determined for a range of crops as this helps gauge the effect of environment on micronutrient concentration and whether the genetic component was sufficient to allow for a breeding initiative to be successful. In some cases (i.e., wheat), the high Zn trait was found in wild relatives, and this created a more complex strategy to first bring in the high Zn alleles across from the wild relatives, such as the making of synthetics which could then be used in the conventional breeding program. As of 2016, HarvestPlus partners had released more than 140 biofortified varieties of 10 staple crops in 26 countries (Andersson et al. 2017).

The progress to date is significant, with 340 biofortified varieties from 12 crops having been released in 40 countries (Fig. 1) and potentially a broader range of crop varieties undergoing testing in various target countries (Fig. 2) (Bouis et al. 2020).

Micronutrient screening

Accurate and precise micronutrient analysis is essential for a successful biofortification breeding program and major advances in screening for nutrient-dense crops have been realised over the last

20 years. Colorimetric methods are used as a rapid visual tool for screening grain samples in the preliminary stages of the breeding process.

Direct association between the ICP and colorimetric method measurements for Fe was seen in wheat, rice, and maize and for Zn in wheat (Choi et al. 2007; Ozturk et al. 2006).

Colorimetric methods are semi-quantitative and therefore are good for screening, but the preparation and staining can be a laborious task and not entirely suitable for a breeding program. Atomic absorption spectrometry (AAS) and Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) or Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) are optimal analytical methods that give total nutrient levels but are expensive analyses and require high purity chemicals, so are not ideal outside of a very clean laboratory. Energy Dispersive X-Ray Fluorescence (EDXRF) is a low cost, stable, non-destructive, high-throughput, multi-elemental method that requires little or no sample preparation and is appropriate for measuring a wide range of Fe and Zn concentrations.

XRF results are highly correlated with ICP-MS analysis in wheat (Paltridge et al. 2012a), rice, pearl millet (Paltridge et al. 2012b), maize and bean (Guild et al. 2017). EDXRF has also been shown as a successful screening technique for the HarvestPlus secondary crops cowpea (Guild et al. 2017), potato (Sosa et al. 2018), lentil and sorghum (Guild and Stangoulis 2021). Being a fast (30–60s per sample) and cheap (less than a US \$2 per sample) analytical method with comprehensively described calibration protocol, developed and optimized custom calibrations for specific minerals, XRF has become the widely used method for quantifying Fe and Zn levels in plant samples across various breeding programs. The only disadvantage of this technique is the inability to detect appreciable amounts of the soil contaminants, Al and Ti, so processing from sampling to analysis should be performed with caution (Stangoulis and Sison 2008).

Going forward, the challenge of maintaining accurate and precise analysis will remain and one can see the need for continual validation of Fe and Zn-dense varieties in certified laboratories offering analysis. This will be important to ensure quality control. Rapid analysis with mobile, portable XRFs will be a future technology embraced by the consumer market.

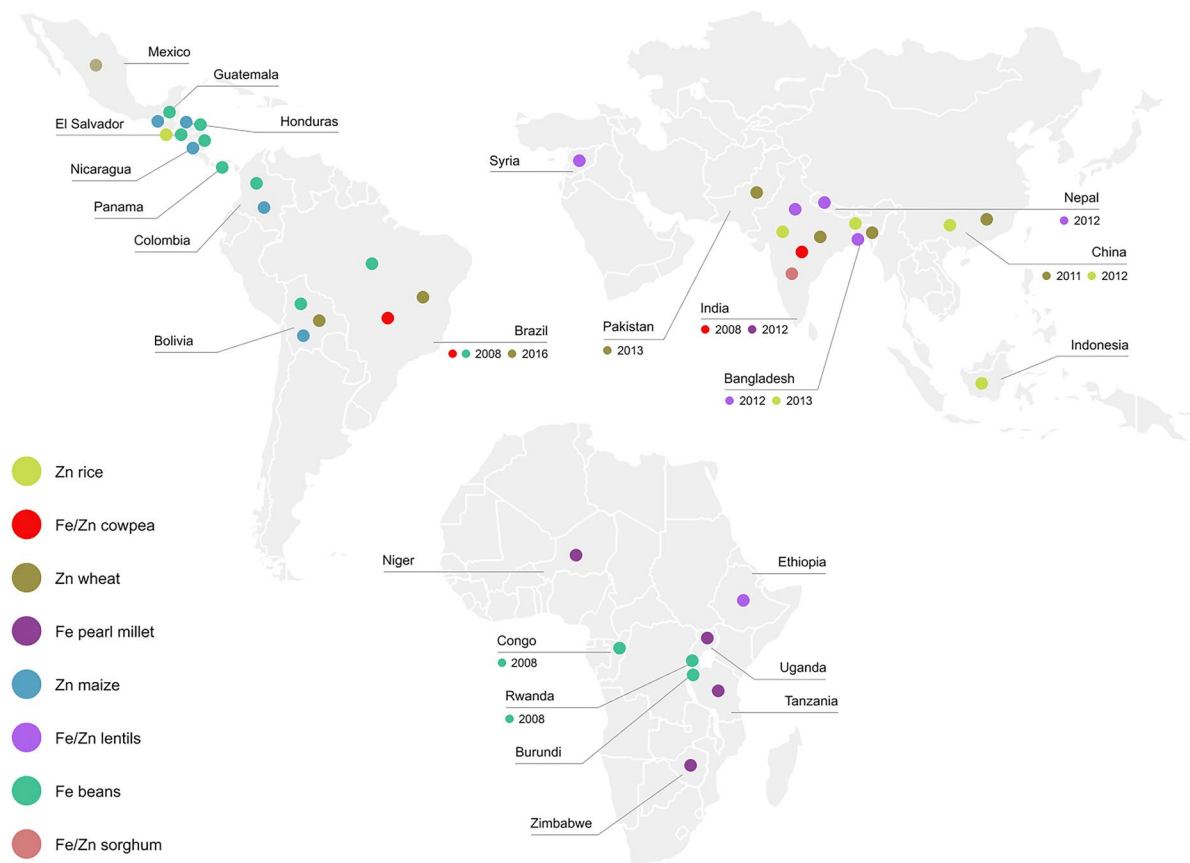


Fig. 1 Fe and Zn biofortified crop varieties released in various countries. Years of the first time released Zn and Fe biofortified crops in certain countries are presented. Figure

constructed based on data provided by (Raney et al. 2013; Andersson et al. 2017; Bouis et al. 2020)

Molecular markers

In recent years, we have seen a significant increase in the number of journal articles that identify QTL for high Fe and Zn (Garcia-Oliveira et al. 2018) and references therein. Marker Assisted Selection (MAS) aims to accelerate cultivar development (Shivay and Prasad 2012). The approach has been effective for other traits in several crop development programs, including tolerance to cold, salinity and drought (Kosová et al. 2014). Molecular marker technology was successful for tracking introgression of related genes underlying a particular trait, for identifying potential genes for nitrogen and nutrient use efficacy, genes involved in the regulation of homeostasis and Fe/Zn incorporation pathways (Chandra et al. 2020). While there are many reports of QTL for Zn and Fe,

the parents used in double haploids or other diversity panels may not have a broad and stable range of Fe and/or Zn and with a large QTL spanning a large segment of a chromosome, it is very hard to identify candidate genes. Genome wide association studies (GWAS) can accelerate development of genetic markers and help in faster identification of genes for biofortification. Gene families involved in Fe and Zn transport are multigene families; various genes are expected to be involved in Fe and Zn uptake, transport and accumulation, so it is important to identify the most suitable gene family members for biofortification of major crops (Alomari et al. 2019).

Genome-wide association studies include a broader genetic base, they are able to identify many trait marker associations and are used to separate the genetics of diverse populations. Thirty-nine

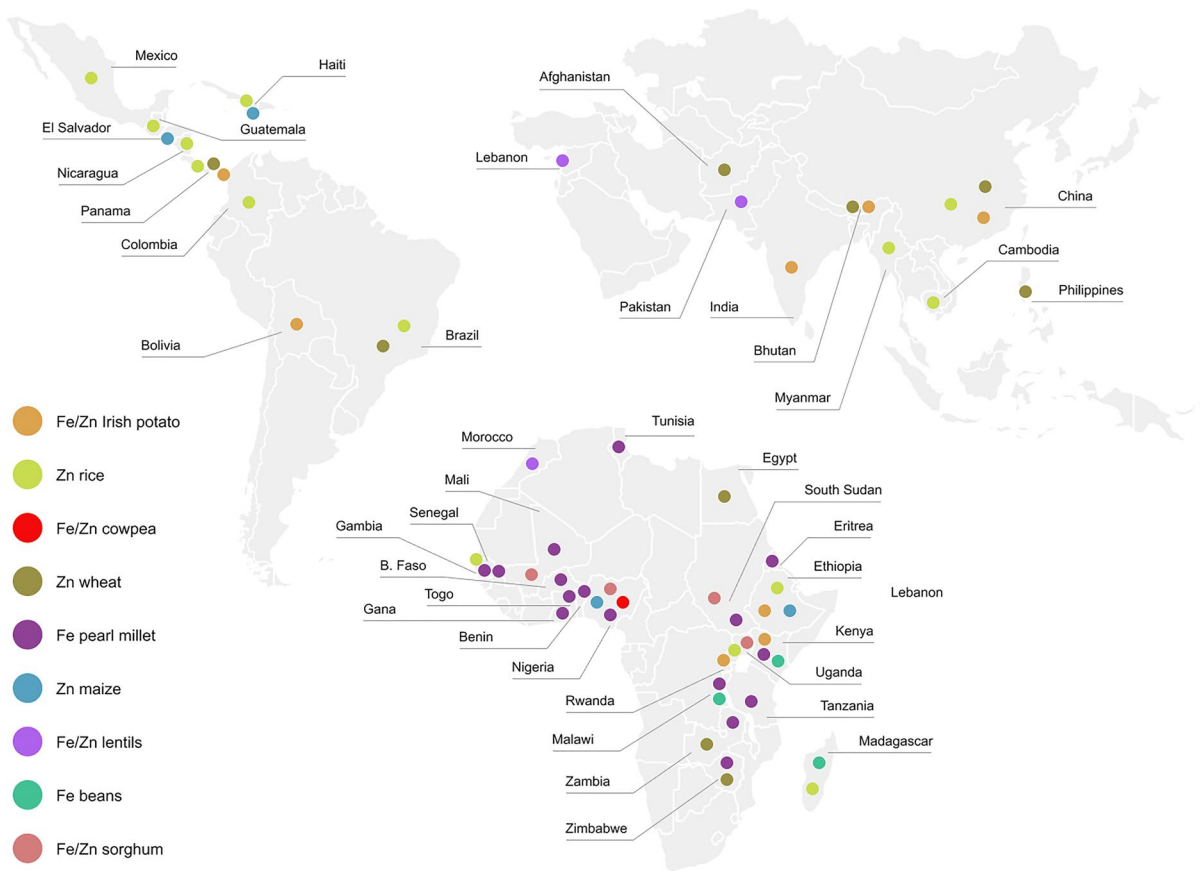


Fig. 2 Currently evaluated Fe and Zn biofortified crop varieties in several countries. Figure constructed based on data provided by Bouis et al. 2020

marker trait associations were identified for Zn on chromosomes 2 and 7 (Velu et al. 2018b). As Fe and Zn concentration of crops is controlled by a large number of loci, it is currently challenging to implement the findings in breeding programs, so more precision is required to discover the exact regions responsible for Fe and Zn concentration and to give an indication of variation in these markers and their potential effectiveness in breeding (Tong et al. 2020). The complete haplotype-block based method could be more effective and practical (Ali and Borrill 2020).

Recent advances in GWAS have allowed for the genetic imputation to increase marker density and resolution (Wang et al. 2018) and this has allowed for a more accurate identification of candidate genes (Wu and Hu 2021). The use of genomic selection

shows great promise for identifying genotypes in breeding programs with moderate to high prediction accuracy for Fe and Zn reported across environments (Velu et al. 2016). One can see this technology more readily used in the future.

The complexity of Fe and Zn transport to fruit, seeds and tubers

There are several major regulatory hurdles for Fe and Zn transport from between the plant root to the shoot and developing fruit, seed or tuber, and these are well covered in several reviews (Bashir et al. 2019; Palmgren et al. 2008; White and Broadley 2011; Puig and Peñarrubia 2009). For root uptake, in brief, plants usually fall into two groups for Fe uptake; Strategy I

(eudicots) and Strategy II (grasses), where the former utilizes a membrane-bound reductase to reduce Fe^{3+} to Fe^{2+} , while in the latter we have the efflux of phytosiderophores such as 2'-deoxymugineic acid into the rhizosphere where it complexes to Fe^{3+} and the phytosiderophore- Fe^{3+} complex is then transported across the plasma membrane into the cytoplasm (Mori 1999). While eudicots and grasses (and cereals of interest in biofortification) appear to fall within the two strategy classes, to date, we seem to have only one known exception to this, where rice also exhibits the Strategy I mechanism, with OsIRT1 and OsIRT2 being the transporters involved in transporting Fe^{2+} across the plasma membrane (Ishimaru et al. 2006). For Zn uptake, it can be taken up by root cells as either Zn^{2+} or as a Zn-phytosiderophore complex. A more detailed description of the transport processes to transport in the xylem and phloem is given in the references above and we will mainly discuss areas where further research is required.

In their review of the physiological limits to Zn biofortification of edible crops, White and Broadley (2011) conclude that Zn concentrations in fruit, seeds and tubers are severely limited by Zn transport in the phloem and there must be an effective way of sequestering the additional metal away so that there are no adverse perturbations in the cytoplasm. Likewise, Fe retranslocation in the phloem is also a limitation in the delivery to fruit, seeds and tubers. Pearson et al. (1996) were able to show good remobilization of Zn in wheat, while Garnett and Graham (2005) reported good remobilization of Fe from shoot to grain in well drained wheat, while in contrast, Hocking (1994) reported a very low level of remobilization of Fe in irrigated wheat, highlighting the effect of environment. Rice also shows poor remobilization of Fe (Marr et al. 1995), while in beans (Zhang et al. 1995) and peas (Grusak 1995) there appears to be good Fe remobilization. There is a clear need to better understand why species variation exists for remobilization, and this will be discussed in subsequent sections.

The activation of senescence is the trigger for remobilization of metal ions to the developing caryopsis and in wheat, the NAM-B1 gene has been implicated in this process as it is a part of the mechanism that stimulates remobilization of metal ions from the vegetative tissues and their directional transport to the developing seed (Waters et al. 2009). The on-going feed-forward control of

seed nutrient loading into seeds is thought to be regulated by nutrient levels reaching the developing seed, however the feedback control generated by filial demand is less well understood. It is thought to involve transmission of regulatory signals from sites of metabolism/compartimentalisation within developing filial tissues. These signals then control nutrient transport and transfer processes located along the source/path/sink system (Zhang et al. 2007). This control could be open to and mediated by signals outside of nutrient flows (i.e., hormones), or closed and mediated directly by plant tissue nutrient levels.

Currently, we know little about the relationship between hormone signalling and micronutrient supply from source to sink. We do know that auxin concentration in developing grain correlates with grain filling rate in rice (Xu et al. 2007) and wheat (Liu et al. 2013) with cytokinin levels also showing positive links to maximum grain weight and grain filling rates in wheat (Liu et al. 2013). There is a need for more research in this area.

As an example of a closed loop system that's mediated by nutrient levels, there is the increase in filial demand for Fe by the expression of soybean ferritin in rice seed, leading to increased Fe deposition within the endosperm (Qu et al. 2005). Zhang et al. (2007) hypothesised that the increased supply of Fe may be derived from source tissues after a signalling pathway is activated by depletion of Fe in the filial cytoplasm. Again, more information on the signalling pathway is required for a targeted approach in plant breeding.

Genotypic variation in filial growth rates appears to be linked to differences in Zn accumulation within wheat (Stomph et al. 2011). Grain protein levels have also been implicated in providing the sink strength (Kutman et al. 2011) and delivery of amino acids is also dependent on their delivery via the phloem. We know little about the proteins binding Zn and Fe within sink tissues and more definitive studies are needed in this area. (Hegelund et al. 2012) reported metallothionein's in barley filial tissues and they are known to bind Zn. A question arises as to whether natural variation exists for Zn and Fe binding proteins and whether this could help to explain subtle differences in Fe and Zn content. Furthermore, whether they could be chemical species for further genetic manipulation to help improve the bioavailability of the nutrients.

Abiotic and biotic stressors negatively impact on photosynthetic activity and sucrose levels in leaves (Rosa et al. 2009), and this in turn impacts on the delivery of both photo-assimilate and micronutrients to developing seeds. Also, with climate change, there is a greater incidence of extreme events (Lobell and Gourdjji 2012), including heat and drought stress (Lobell et al. 2012), and this has the potential to impact on nutrient uptake and nutrient delivery to fruits, seeds and tubers.

There is a need to better understand the complex phloem signalling relationship and to identify genotypes with efficiency and tolerance mechanisms that lead to maintaining adequate carbon fixation and phloem photoassimilate/micronutrient levels.

There are commonalities between cereal species targeted for biofortification when it comes to long distance transport. Simply, xylem is unidirectional and will provide the water and nutrients while the phloem is bidirectional and delivers photoassimilate and nutrients to various tissues to maintain growth and metabolism. Plant structural differences do exist between species and even within a species we have differences in growth and nutrient requirements. One such anomaly found between species is the xylem discontinuity (Thorne 1985; Zee and O'Brien 1970).

Fundamentally, a nutrient entering the seed must first be transported from the xylem to the phloem before it is translocated to the developing reproductive tissues of crops such as wheat, barley and oats. Rice, however, has a continuous xylem and the vascular bundle is located around the circumference of the seed (Thorne 1985). Therefore, the long-distance transport characteristics in rice are dissimilar to many of the other cereals. In rice, there is a direct route (for root derived Zn) transported to the developing caryopsis via the xylem, coupled with xylem to phloem transfer at stem and panicle nodes (Nishiyama et al. 2013; Yoneyama et al. 2010). Also, unlike other cereals, rice has a mechanism of relatively uniform deposition of Zn throughout the endosperm, the loss due to polishing is around only 10–15% of total grain Zn (Jaksomsak et al. 2018). In contrast, Fe in rice mostly accumulates in the aleurone layers and embryo (Kyriacou et al. 2014). Structural anomalies within the seed also exist and appear to influence the Fe and Zn distribution within the seed. In wheat and barley, the grain crease runs the length of the seed kernel, and a single vascular bundle is embedded within the

maternal pericarp. In rice, there is a singular vascular bundle embedded within the pericarp, but nearer the outer grain surface (Thorne 1985). Wheat and rice unload assimilates over the full length of the bundle and when given excess Zn, in wheat it accumulates this excess Zn in the crease region (Stomph et al. 2011). A transport barrier to the endosperm appears to be present at the endosperm cavity. In rice, the vascular bundle is located around the circumference of the seed and on unloading, there is symplastic transport through the plasmadesmata, to the nucellus and transport into the aleurone and then the endosperm. The endosperm cavity is not present, and this is one less obstacle for the movement of nutrient. Also, in close proximity to the phloem in rice is the xylem vessel, and what role that plays in the delivery of Zn (in particular) needs further research.

In the rice phloem, Fe and Zn chelate mostly to 2'-deoxymugineic acid and nicotianamine, respectively (Nishiyama et al. 2012). There is some suggestion that not all rice genotypes can remobilise Zn (Impa et al. 2013), where the authors found that under Zn-sufficient conditions there was continued root uptake during the grain filling stage and this was the major source of Zn unloading into the rice grains, a finding also supported by (Jiang et al. 2007), whereas under Zn deficiency, a number of genotypes remobilised Zn from shoot to root and then back to the grain, but not all genotypes did this.

Further physiological studies across a broader range of genotypes that differ in their seed Zn at maturity is required as this may aid in plant breeding.

The future potential of biotechnology

Modern biotechnology tools can be used for the development of biofortified crops. Low-cost, high-speed technologies that enable the discovery of non-targeted genes and alleles have been established (Carvalho and Vasconcelos 2013).

Various genomics and molecular breeding approaches i.e., marker-assisted selection, high-throughput phenotyping, and genotyping for selecting particular traits could be incorporated into genetic engineering and breeding programs designed for Fe and Zn biofortification strategies (Ricroch and Hénard-Damave 2016). Reverse-breeding, oligo-directed mutagenesis, 'genome editing' - sequence-specific nuclease technology, and high-throughput

Fe / Zn Biofortification Approaches

Genetic Biofortification

- Conventional plant breeding
- Mutation breeding, through both chemical induction and oligo-directed mutagenesis
- Marker - assisted breeding, including GWAS and the use of KASP markers
- Genomic Selection
- Genome Editing, using CRISPR/Cas 9 technology

Agronomic Approaches

- Mycorrhizal inoculation, AMF
- Fertilizers
- Foliar Fe/Zn fertilization
- Optimizing nitrogen fertilizer application
- Crop rotations

Natural Genetic Variation

- Stimulating efficient uptake of nutrients from the rhizosphere, growth-promoting bacteria (rhizobacteria)
- Increasing Fe/Zn storage and transport
- Enhancing translocation within the plant
- Improving storage of nutrients in the endosperm
- Improving grain mineral bioavailability

Transgenic Approaches

- Reduction of antinutrients
- Overexpression of Fe/Zn transcription factors responsible for homeostasis
- Overexpression of enzymes for the synthesis of nicotianamine and 2'-deoxymugineic acid
- Endosperm specific expression of genes

Fig. 3 Summary of strategies used for Fe and Zn biofortification of crop plants

phenotyping are all-new, modern technologies (Fig. 3) that could provide significant advances in micronutrient biofortification strategies by allowing mixing increased concentrations of minerals and/or promotive compounds with decreased levels of anti-nutrients (Vasconcelos et al. 2017).

Genetic engineering has a role to play in improving the nutrition of the edible portion of a cultivar, by allowing manipulation of a particular gene. Certain transporters/gene families were used to produce transgenics for Fe and Zn biofortification in rice and wheat (Trijatmiko et al. 2016; Singh et al. 2017). New Zn enriched biofortified wheat cultivar, BARI Gom 33, resistant to wheat blast and other common diseases, was recently produced (Barma et al. 2019). Expression of *OshMA1* has an important role in Zn transportation, root to shoot translocation, and is highly upregulated in shoot tissues under Zn deficiency (Li et al. 2015). *OshMA1* expression was seven times higher in Zn-deficient tissues, compared to Zn-sufficient conditions (Suzuki et al. 2012). *OsNAC5* and *OsIDEF1* genes play a role in metal remobilisation, and they are overexpressed for improving Fe concentrations (Kobayashi et al. 2010; Ricachenevsky et al. 2013). Expression of

rice nicotianamine synthase (*OsNAS2*) and soybean ferritin (*SferH-1*) genes contributed to endosperm Fe and Zn enhancement (Johnson et al. 2011; Trijatmiko et al. 2016) and this was achievable in controlled field grown conditions, in a research station (Trijatmiko et al. 2016). Overexpression of *NAS* was also linked to overexpression of Fe transporters (Boonyaves et al. 2017).

Transgenic rice lines overexpressing the Fe (II)-nicotianamine transporter *OsYSL2* and Fe storage protein ferritin contained higher concentrations of both Zn and Fe, 1.6- and 6-fold increase, respectively. The introduction of several genes was much more efficacious in increasing the Fe and Zn concentrations than the inclusion of a single gene (Masuda et al. 2012). The over-expression of ferritin increased Fe accumulation in rice and wheat grain seeds even when plants were grown under Fe deficient conditions (Borg et al. 2012; Masuda et al. 2013).

Interestingly, an increased expression of Fe transport and storage proteins improved both Fe and Zn concentrations in transgenic rice, 3.4 and 1.3 folds, correspondingly (Aung et al. 2013). A two-fold increase in Fe concentrations was seen when

a vascular Fe transporter was expressed under an endosperm-specific promoter (Connorton et al. 2017).

Transgenic high yielding Indica rice with an expressed ferritin gene from soybean provided a 2.54 increase in Fe and 1.54 increase in Zn concentration (Paul et al. 2014). Transgenic rice plants that expressed 2'-deoxymugenic acid (DMA) and nicotianamine collected up to 4-fold more Fe and 2-fold more Zn in the endosperm (Banakar et al. 2017).

While the enrichment of grain endosperm with Fe and Zn by the use of molecular strategies is becoming increasingly popular within the research community, biotechnological tools to increase not only the concentration but also the bioavailability of minerals in crops needs consideration.

Bioavailability refers to the relative amount of a micronutrient that is absorbed in the human gut from a certain crop plant, and it depends on several external and internal factors and intestinal digestion, so the exact determination of bioavailability of a nutrient in a plant part is not a straightforward process. In crops, 80% of phosphate is in the form of phytic acid accumulated in the seeds (Bohn et al. 2008). Phytic acid is a strong indicator of bioavailability, a ratio of <1:1 phytic acid: Fe, without any enhancers of Fe absorption, or phytic acid: Zn <1:15 is needed for improved absorption of Fe/Zn from crop plants (Gibson 2006; Hurrell and Egli 2010). However, the bioavailability of micronutrients is not entirely attributed to phytic acid levels as in certain crops QTL for total Fe concentration in grain is overlapping with the QTL for Fe bioavailability in grain (Lung'aho et al. 2011). Similarly, it was shown that increasing the content of NA or overexpression of NAS gene enhances Fe bioavailability in wheat and maize (Beasley et al. 2019). Overexpression of NAS is shown to be a promising approach for improving bioavailability of Fe and Zn (Clemens 2019). High throughput technologies can be used in this instance and could produce much faster and more efficient effects (Wang et al. 2014). For instance, during heating procedures, Fe (III) hydroxide in ferritin is transformed to Fe²⁺ or Fe³⁺ and chelated with phytates in the food matrix (Hoppler et al. 2008).

Exome sequencing of Fe and Zn homeostasis-linked genes in germplasm, transcriptome profiling, and genome engineering technologies could be used to obtain data on the best possible allelic variants of

target genes to design novel varieties of biofortified crops (Abdallah et al. 2015; Vasconcelos et al. 2017). Extended shelf life, enhanced yield, improved nutritional value and decreased antinutritional factors in crops are agronomic traits that could be modified with genome editing (Abdallah et al. 2015). New technologies allow for biofortification of amino-acids and manipulations in fatty acid content of crop grains (Che et al. 2016).

The success of agronomic research

Foliar fertiliser application is generally seen as a way of overcoming low grain nutrient concentrations caused by immobilization of a mineral in the soil (Fig. 3) (Duxbury et al. 2006). Through leaves, plants absorb Fe and Zn and in theory that would lead to improved absorption at the point of utilization; in growing tissues (Khoshgoftarmansh et al. 2010). Within the stem and leaf tissue, nutrients can then be transported via phloem or xylem (Rengel et al. 1999) and application at specific plant growth times can improve grain Fe/Zn content and enhance yield when deficiency is present (Cakmak et al. 2010a; Cakmak and Kutman 2018; Farooq et al. 2012; Graham et al. 1992; Graham et al. 1997; Graham et al. 2007; Kiran et al. 2021). Modification of the soil properties, by using animal manure and plant residues has also been shown to increase Fe and Zn availability (Prasad et al. 2014) but foliar application appears to be much more effective than non-foliar, soil nutrient application in the enhancement of grain concentrations of nutrients. Yet, the effectiveness of foliar application is crop specific, with rice showing site differences in the efficacy of foliar applied Zn (Prom-U-Thai et al. 2020; Wei et al. 2012), whereas for wheat, foliar applications of Zn is very effective in increasing grain Zn across different environments. For example, a Zn foliar application of 0.5% ZnSO₄ increased Zn concentration of wheat grains by 95% and bioavailability by 74% (Hussain et al. 2012). Increased transport of nutrients from leaves into seeds was attained via spraying 0.5% Zn solution and grain Zn concentration doubled (Peck et al. 2008). Similarly, the application of Fe fertilisers via foliar feeding enhanced Fe uptake and translocation (Velu et al. 2014; Wei et al. 2012). Different fertilizers, both organic and inorganic Zn/Fe salts have a crucial role in the

transport of micronutrients from leaves to the grain (Colle et al. 2009). The timing of foliar application is important as it determines the effectiveness of unloading optimal amounts of nutrient into grains (Cakmak et al. 2010b). In wheat, the milk to grain filling stages are the most effective periods for biofortifying grains with Zn (Cakmak et al. 2010b; Ozturk et al. 2006; Yang et al. 2011), with good enrichment predominantly in the aleurone and embryo (Cakmak et al. 2010b). Foliar applications of fertiliser in the grain maturation stage, near harvesting appears to be very efficient as starchy endosperm absorption is up to three-fold and the phytate concentration is at a sub-optimal level (Velu et al. 2014). Das et al. (2019) reported that an optimal time for increasing grain mineral concentration was achieved with a combination of foliar application of Zn after anthesis, plus a soil mineral application (Das et al. 2019). Knez et al. (2018) developed Zn biofortified wheat grain by a foliar application of 1.5 L ha⁻¹ ZnSO₄ during the mid-vegetative growth stage followed by 2 L ha⁻¹ ZnSO₄ two weeks after flowering. The mid-vegetative application was used to mitigate any effect of Zn deficiency, a common occurrence in the high pH soils of the Wimmera region of Victoria, Australia. Interestingly, the high Zn flour had a Zn and Fe concentration of 47.2 mg kg⁻¹ and 58 mg kg⁻¹ respectively, while the low Zn flour had 33.6 mg kg⁻¹ and 53 mg kg⁻¹ correspondingly, indicating that in wheat, and given careful consideration of fertiliser application timing, one can not only raise the levels of Zn but also Fe. This differential in Zn was sufficient to affect Zn bioavailability and the Zn status of the chickens in a feed study (Knez et al. 2018) that also altered the gut microbiome (Reed et al. 2018). Optimal times in applying foliar sprays of Zn appear to be closely linked to optimal concentration in the phloem feeding the developing wheat caryopsis, that being around 9–21 days after anthesis (Palmer et al. 2014).

The effectiveness of biofortified products to improve Zn nutritional status of animal (including human) subjects has been tested over the years. Welch et al. (2005) were the first to demonstrate the beneficial effect of Zn biofortified wheat in improving Zn status of rats. Comparable results were reported by Rosado et al. (2009) in humans and Carlson et al. (2012) in pigs, with the highest concentration of Zn

seen in subjects fed the diets with the highest Zn concentration; consumption of Zn biofortified varieties of certain crops improved Zn status.

Similar findings were provided for Fe biofortified crops, consumption of Fe enriched foods improved Fe levels of consumers. In addition, protective effects of Fe biofortified foods on animal gut microbiota were shown, provision of Fe biofortified crops increased the presence of beneficial and decreased the abundance of potentially pathogenic bacteria in the gut (Reed et al. 2017; Reed et al. 2018; Morais-Dias et al. 2018; Beasley et al. 2020).

Challenges - soil deficiency

The bioavailability of a nutrient in the rhizosphere is well studied and in part is affected by clay content, cation exchange capacity, the content of soil organic matter, and soil pH (Marschner 2012; Nguyen and Marschner 2017; Ramzan et al. 2014). Arguably, the most widespread micronutrient deficiency in the world today is Zn deficiency, observed in more than 50% of investigated soils with deficiency rates ranging from 48% up to 86% (Dharejo et al. 2011; Manyevere et al. 2017; Reza et al. 2017; Shukla et al. 2017; Singh et al. 2005). Only 10% of Zn is available to plants (Alloway 2009).

Zn and Fe concentrations in the grains are strongly linked to the inherent Zn and Fe capacity of the soils, so a suitable level of water and minerals in the soil during the reproductive stage of plant development is needed for adequate grain nutrient concentrations (Cakmak and Kutman 2018). An indirect correlation is seen between Fe and Zn and cation exchange capacity of the soil (Yoo and James 2002). The higher the exchange capacity, the lower the availability of Fe and Zn.

Native soil Zn status and its physiochemical properties are determining factors of rice grain Zn concentrations (Wissuwa et al. 2008). Rice grain Zn concentrations could range from 8 to 47 mg/kg, depending on the soil status. The use of soil applied fertilizers cannot compensate for the low accessibility of Zn in the soil, while the addition of fertilizers increased straw Zn concentrations and improved straw and grain yield, but grain Zn concentrations remained unchanged or were only slightly elevated,

irrespective of genotype (Duxbury et al. 2006; Wisuwa et al. 2008). An application of Zn as a foliar spray during the early-grain filling stage could be helpful as it has been shown that it increased Zn concentrations from 3.7 to 5.6-fold in peas and Zn concentration to 60 mg Zn/kg (Poblaciones and Rengel 2016). High concentrations of Zn in roots and leaves can be accomplished with soil Zn fertilizers (Wei et al. 2012).

The rhizosphere effect and biofortification

The uptake of Fe and Zn into the root and their long-distance transport have been extensively reviewed over recent years (Palmgren et al. 2008; Puig and Peñarrubia 2009; White and Broadley 2011) and we are well aware of the importance of the rhizosphere in the supply of Fe and Zn for root uptake.

The rhizosphere environment is dynamic and has a direct influence on plant growth and yield (Hakim et al. 2021). Basically, a Zn/Fe deficient rhizosphere gives nutrient deficient plants (Welch and Graham 2005), and the effect of soil textural properties greatly impacts on the availability of Fe and Zn in the soil solution, and hence the availability for scavenging by plant roots. Having a role in nutrient availability and maintenance of plant health, is the microbiome (Berendsen et al. 2012; Lehmann et al. 2014) and this will be discussed in more detail as it is an area that shows potential for genetic manipulation to improve nutrient availability and uptake into the edible portion of the crop plant.

Growth-promoting bacteria are essential components of the rhizosphere (Chandra et al. 2020). Root colonizing rhizobacteria have been shown to stimulate plant growth, grain protein content, yield, and nutrient uptake in plants (Rodrigues et al. 2008; Sharma et al. 2011). Their presence improves the soil structure, aeration, and fertility, suppresses the growth of pathogens, and improves plant immunity (Hakim et al. 2021). Inoculation of soils or crops with rhizospheric microbes is shown to enhance the content of Fe and Zn in plant tissues (Ku et al. 2019).

Microorganisms within the rhizosphere help to provide soluble forms of essential nutrients (Szymańska et al. 2016). Additionally, plant growth-promoting rhizobacteria and endophytic bacteria contribute to growth-promoting activities such as the formation of

root nodules, solubilization of minerals, reduction of negative effects of certain pathogens, phytohormone production, and degradation of xenobiotic compounds (Chandra et al. 2020). Rhizospheric microbes can be a useful strategy for increasing the concentrations of micronutrients in edible parts of crops plants. Further research is required to explore the physico-chemical characteristics of the rhizosphere, to identify new microbial communities and to explore plant-nutrient-rhizobacteria interaction.

The effect of mycorrhizal inoculation is also of interest as an agronomic intervention strategy to improve the nutrition of food products, such as seeds. With widespread nutrient deficiencies throughout many of the world's arable soils, mycorrhiza could help mitigate sub-optimal supply of P and micronutrients such as Zn. In a comprehensive meta-analysis on 104 articles comprising 263 trials to test the influence of 10 independent variables on arbuscular mycorrhizal fungi (AMF) - mediated Zn concentration, in comparison to non-mycorrhizal controls, AMF had a positive overall impact on Zn concentration in above ground plant tissues, fruit and seed tissue (Lehmann et al. 2014). A very clear impact of mycorrhiza was reported by (Ercoli et al. 2017), showing an effect of AMF inoculation on Fe and Zn in field grown durum wheat. (Lehmann et al. 2014) in their meta-analysis identified soil texture as modulating AMF, with soil pH and soil Zn concentration affecting AMF-mediated shoot Zn concentration and soil P, influencing fruit Zn concentration. The environmental conditions therefore play a major role in the efficacy of AMF inoculation. Variable gains by AMF as witnessed in wheat and barley by (Coccina et al. 2019) could also be explained by choosing genotypes with variable efficiency in phloem retranslocation and unloading into the caryopsis. The authors found that the mycorrhizal pathway of Zn uptake contributed up to 24.3% of total above-ground Zn in wheat, and up to 12.7% of that Zn in barley. Their results suggest that AMF has a substantial role in uptake of Zn into cereals and the proportional contribution by the mycorrhizal pathway of uptake is dependent on plant species, as well as available soil Zn. Similarly, a recently published paper by Yazici et al. (2021) showed that AMF impairments decreased shoot and grain Zn concentrations and increased cadmium build-up in wheat plants while mycorrhizal replenishment

and colonisation promoted shoot and grain Zn accumulation.

Further research should concentrate on finding out the most efficient methods of mineral application to promote uptake and to maximize the accumulation of Fe/Zn in all edible parts of crop plants. Microbial and genomic assisted agronomic strategies may provide a long-lasting solution, but this entails further investigation.

Nitrogen application results in enhanced nutrient deposition in grains

Grain quality is strongly linked to the availability of higher doses of N in the late reproductive phase of plant development, and a plant's N status influences the uptake, transport, and relocation of nutrients within the plant tissue (Palmer and Guerinot 2009). Increased N levels improve the root uptake and passage of Zn, consequently increasing the deposition of Zn in seeds (Cakmak et al. 2010c). A link to an effect of N on phytosiderophore release has been made, with an increase in N supply being very effective in improving the release of phytosiderophores from roots, improving the mobilisation, root uptake, and shoot transport of Fe in Fe deficient wheat plants (Aciksoz et al. 2011). Phloem loading, phloem to xylem exchange, deposition of nutrient into grain, depends on nutrient transporter proteins, which activity is enhanced by the addition of N (Palmer and Guerinot 2009). Also, with increased N application, an enhanced synthesis of long-distance transport compounds such as nicotianamine and 2'-deoxymugineic acid would provide the chelate for long-distance transport. A study that utilizes appropriate phloem collection techniques, such as aphid stylectomy, coupled with metabolite analysis, would be beneficial in further understanding the effect of elevated N on increased Zn deposition in grains.

Crop rotations to maintain a biofortified crop

Crop rotation methods are an integral part of sustainable agriculture, however limited information is available on their role in the quality of the edible portion of the crop, including the nutrient concentration. If considering the effect of N on seed Zn and Fe (see previous section), then a role for rotation in maintaining N

supply to crop plants and thereby maintaining a balanced nutrition, is a worthwhile consideration.

Cereal-cereal rotations have been shown to cause nutrient imbalance, soil degradation, and a deterioration in crop productivity (Chauhan et al. 2012). In contrast, diverse crop rotations help enhance soil quality by improving soil structure, levels of soil organic matter, and mycorrhizal milieu (Hobbs et al. 2008; Plenchette et al. 2005). Similarly, rotations improve plant productivity by increasing nutrient use efficiency and the general quality of grains (Mady Kaye et al. 2007). The incorporation of leguminous plants in crop rotations is an efficient practice for improving the nitrogen cycle in conventional breeding systems (Kubota et al. 2018). Meta-analysis data show that a replacement of bare fallow with legume crops provides benefits as rotation decreases nitrate leakage by 40% (Tonitto et al. 2006). When legumes were included in rotations, the nitrogen (N) input of subsequently grown crops was much higher (St. Luce et al. 2016). Similarly, post-harvest cover crops are preserving satisfactory soil environments, the use of two winter cover crops in summer crop farming regions improved the yield and N uptake (Gabriel et al. 2016). Diversification with pulse compost increased water accessibility for subsequent crops (Gan et al. 2015).

The inclusion of pulses in wheat-maize rotation improved soil organic carbon and bioavailability of micronutrients (Venkatesh et al. 2017). Pulses could be a suitable solution for modification and escalation of cereal-cereal rotations. The inclusion of pulses positively affects the nutrient cycle in the soil-plant system due to its impact on soil residual fertility and acquisition of minerals by subsequent crops (Venkatesh et al. 2017).

The advantages of raw and forage crop rotation on yield and mineral concentration in maize showed that two years rotation with maize-soybean-spring wheat and three years rotation with maize-soybean-oat/pea hay plus alfalfa increased yield and kernel phosphorous and potassium concentrations (Riedell and Osborne 2017). Rotations with deep-rooted forage crops i.e., alfalfa increased N availability and the yield of shallow-rooted crops like maize (Ma and Zheng 2018; Riedell et al. 2009).

The sustainable production of crops requires an improved understanding of the potential beneficial effects of diverse crop rotations. New strategies

for improving soil health, sustainability, and crop throughput via crop rotation mechanisms merit additional attention and further work.

How to introduce Fe and Zn-dense biofortified varieties?

A major challenge in the future is maintaining high Fe and Zn in a biofortified variety, once released to farmers. A reduction in concentration can happen for various reasons, including the mixing of seed (biofortified and non-biofortified) and poor agronomic practices, including fertilizer management. Biotic and abiotic stress can also impact on final Fe and Zn concentrations in the edible portion of a crop, and linked to this is research of climate change effects on seed nutrition that also indicate significant changes (Myers et al. 2014). A recent failure of biofortified beans to reach levels of Fe above locally grown varieties sourced from the market-place in East Africa (Glahn et al. 2020), is further evidence that there is a need to continually monitor biofortified varieties and to provide ways to mitigate perturbations in nutrient concentration. There is obviously a need to better understand local farmer seed systems.

In the marketplace, Fe and Zn concentration can be monitored using rapid analysis devices, such as portable XRF instruments. Plant breeders have developed a biofortified variety based on a *tried-and-true* formula of sourcing Zn and Fe-dense progenitors and crossing to parents of good agronomic performance, developing large populations and through selection, maintaining nutrient-dense genotypes through the generations. Breeders also conduct multi-location trials and make selections on performance against local checks, but often sites are well maintained, and a farmer may not have the same resources. Irrespective of whether the genetics of a new biofortified variety is sufficient to deliver a stable Fe or Zn end-product, the environment where that biofortified variety grows, can still have an impact on final product concentrations.

From a plant nutrition viewpoint, there are a number of reasons why a biofortified product may not meet its target in a farmer's field and one needs to appreciate the importance of a continued Fe and Zn availability in the soil solution for root uptake and long-distance supply to developing sinks within the plant. Efficiency in root uptake is of paramount

importance, and when one considers the levels of macro and micronutrient deficiency worldwide, many of the target farmers in developing countries are poor and unable to afford costly inputs, to mitigate the effects of deficiency.

Conclusions

To sum up, Fe and Zn enrichment of grains in many crops has already been achieved. Novel biofortification programs and strategies need to be developed to tackle micronutrient insufficiency in crops, particularly considering new environmental constraints. Microbe-mediated biofortification has great potential and warrants further research. New technologies should aim to enhance genotypes that could be used in biofortification programs and to additionally develop techniques for faster breeding, dissemination, and implementation of Fe and Zn enhanced cultivars. Finally, the mineral nutritional quality of food crops should aim to encompass all major macro, micro, and antinutrients. There is a need to integrate more micronutrients and broaden biofortification projects beyond Zn and Fe. Additional regulations are needed to address public safety concerns, ensure adequate monitoring and implementation of transgenes in biofortified crops, and illuminate the effect of transgenic crops on human health. Biofortification efforts of major crop plants should be augmented to respond to new nutrition and health challenges related to the double burden of malnutrition and address the need for diverse and sustainable diets with a maximum beneficial impact around the world. Collaboration between different parties, plant breeders, farmers, consumers, scientists from various disciplines, and national and international organisations and governments is of crucial importance in this instance. Finally, biofortification strategies should be well incorporated in the nutritional agendas so that a vision of reaching 9 billion people by 2030 can turn into a reality.

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