Chapter 9

Biofortification of Staple Crops

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1. INTRODUCTION

Micronutrient malnutrition, also called "hidden hunger", caused by the deficiency of micronutrients in the diet, afflicts more than two billion people worldwide, especially women and preschool age children (Welch and Graham 2002; White and Broadley, 2009) in the developing countries who are largely dependent on staple food crops. The consequences, in terms of malnutrition and health, are devastating and can result in blindness, stunting, diseases, and even death. The major reason for micronutrient deficiency in the populations of third world countries is the predominance of non-diversified cereal and plant-based diets, which are poor in micronutrients, as compared with the meat-rich diets of people in developed countries (FAO, 2004; Grotz and Geurinot, 2006; Gomez-Galera et al., 2010). Moreover, processes like polishing, milling, and pearling of cereals make them even poorer in micronutrients (Welch and Graham, 2004; Borg et al., 2009). Anti-nutritional factors such as phytic acid, fibers, and tannins further reduce the bioavailability of these minerals from dietary intakes by preventing their absorption in the intestine (White and Broadley, 2005; Brinch-Pederson et al., 2007; Pfeiffer and McClafferty, 2007).

1.1 Magnitude and Causes of Micronutrient Malnutrition

Over two billion people in the developing countries suffer from micronutrient malnutrition (Table 9.1). The costs of these deficiencies in terms of lives lost and poor quality of life are staggering. Iron and zinc deficiencies are the most common and widespread, afflicting more than half of the human population (World Health Organization, 2002; White and Broadley, 2009) among which developing countries of Asia and Africa are the most affected (Hotz & Brown, 2004; Gomez-Galera et al., 2010). More than two billion people are iron deficient, and the estimates of zinc deficiency are also near this proportion (ACC/SCN, 2000; Prasad, 2003; Welch and Graham 2002; Gibson, 2006;

Agricultural Sustainability. DOI: http://dx.doi.org/10.1016/B978-0-12-404560-6.00009-5

TABLE 9.1 Level and Effects of Micronutrient Malnutrition

Deficiency	Prevalence in Developing Countries	Groups Most Affected	Consequences
Iron	2 billion people	All, but especially women and children	Reduced cognitive ability; childbirth complications; reduced physical capacity and productivity
Zinc	May be as widespread as iron deficiency	Women and children	Illness from infectious diseases; poor child growth; pregnancy and childbirth complications; reduced birth weight
Vitamin A	250 million children	Children and pregnant women	Increased child and maternal mortality; blindness

Source: ACC/SCN (2000).

Thacher et al., 2006). Iron takes part in most of the redox reactions in the body and also acts as a cofactor in numerous vital enzymatic reactions (Kim and Geurinot, 2007). Likewise, zinc is an essential micronutrient for regulating gene expression and maintaining structural integrity of proteins. It acts as a cofactor in more than three hundred enzymatic reactions (King and Keen, 1999). Deficiencies of iron and zinc lead to poor growth and compromised psychomotor development of children, weakness, fatigue, irritability, hair loss, reduced immunity, wasting of muscles, sterility, morbidity, and even death in acute cases (Prasad et al., 1961; Pfeiffer and McClafferty 2007; Wintergerst et al., 2007; Stein, 2010).

Similarly, approximately three million preschool age children suffer with visible eye damage owing to vitamin A deficiency globally, and an estimated quarter-million to half-million preschool children go blind from this deficiency annually, and about 66% of them die within months (Mason et al., 2001). Even more importantly, the last two decades have brought an awareness that vitamin A is essential for immune functions.

Cereal grains have inherently low concentrations of micronutrients such as Zn and Fe, particularly when grown on micronutrient-deficient soil. In wheat and rice, only a small fraction of iron is transported to the grains from the senescing leaves whereas more than 70% of zinc is mobilized into grains (Grusak and DellaPenna, 1999; Grusak et al., 1999). In cereals these micronutrients are primarily stored in husks, the aleurone layers, and embryos which

are lost during milling and polishing processes (Welch and Graham, 2004; Borg et al., 2009; Lombi et al., 2011). For better nutrition of human beings, Zn or Fe content of wheat grains should be improved to around 40–60 mg/kg, from the existing available amount of 10–30 mg/kg (Cakmak et al., 2000).

1.2 Strategies for Alleviating Micronutrient Malnutrition

To alleviate micronutrient malnutrition, a comprehensive strategy involving various interventions—such as supplementation, dietary diversification, fortification, and biofortification—adapted to conditions in different countries and regions is required (Zimmerman and Hurrel, 2007; Stein, 2010). Supplementation involving the oral delivery of micronutrients in the form of pills and syrups has been used in chronic deficiencies. Fortification is the addition of the desired minerals to food and stuffs like iodine in salts. Recurring expenditure and lack of a robust distribution system and careful implementation are some of the problems associated with these approaches. The most economical and feasible approach to alleviate hidden hunger is biofortification, a strategy for producing staple food cultivars whose edible portions have a higher concentration of bioavailable minerals and vitamins.

2. BIOFORTIFICATION: A NEW TOOL TO REDUCE MICRONUTRIENT MALNUTRITION

Biofortification refers to genetically increasing the bioavailable mineral content of food crops (Bouis, 2000; Brinch-Pederson et al., 2007). Developing biofortified crops also improves their growth and production in soils with depleted or unavailable minerals (Cakmak et al., 2008; Borg et al., 2009). Conventional and molecular breeding and genetic engineering techniques are the approaches that may be used to biofortify the crops with iron, zinc, and vitamin A (DellaPenna, 1999; Johns and Eyzaguirre, 2007; Pfeiffer and McClafferty, 2007; Tiwari et al., 2010).

This approach offers multiple advantages over other approaches. First, it capitalizes on the regular daily intake of a consistent and large amount of food staples by all family members. Second, after one-time investment to develop seeds that fortify themselves, recurrent costs are low, and the germplasm can be shared internationally and will be cost effective. The third advantage is that the biofortified crop system is highly sustainable. Fourth, biofortification provides a feasible means of reaching undernourished populations in remote rural areas, delivering naturally fortified foods to people with limited access to commercially marketed fortified foods. Fifth, breeding for higher trace mineral density in seeds will not incur a yield penalty. With mineral-dense seeds, more seedlings survive and initial growth is more rapid. Ultimately, yields are higher, particularly in trace mineral "deficient" soils of arid and calcareous regions (Monasterio et al., 2007).

HarvestPlus leads the global effort to make the familiar staple foods more nutritious and available to those suffering from hidden hunger. Founded in 2003 it is now a part of the CGIAR Research Program on Agriculture for Nutrition and Health. HarvestPlus uses biofortification to breed higher amounts of vitamins and minerals in staple foods, including bean, cassava, orange sweet potato, rice, maize, pearl millet, and wheat. Its activities are coordinated by the International Center for Tropical Agriculture (CIAT) and the International Food Policy Research Institute (IFPRI). The HarvestPlus biofortification program mainly focuses on three micronutrients—iron, zinc, and vitamin A—that are widely recognized by the World Health Organization (WHO) as limiting.

2.1 Conventional and Molecular Breeding Approaches for Biofortification

During the "green revolution", plant breeding efforts led to enhanced grain productivity of staple cereal crops. Such breeding efforts, along with improved agriculture technologies, succeeded in providing enough calories and protein to prevent the threatened massive starvation and famines predicted in the early 1960s in many regions. Breeding for micronutrient-enriched staple plant foods can be pursued (Bouis, 1996; Graham et al., 1998, 1999; Graham and Welch, 1996) for combating hidden hunger. The predominant cultivars of cereal crops have limited variability for grain iron and zinc concentrations/contents (Cakmak et al., 2000; Graham et al., 2001; Bouis, 2003; Rawat et al., 2009). A wide range of wheat germplasm has been screened at CIMMYT, Mexico for concentration of Fe and Zn in the whole grain over different environments. Graham et al., (1999) reported a range of 28.8–56.5 $\mu g\, g^{-1}$ (mean = 37.2 $\mu g g^{-1}$) for Fe and 25.2–53.3 $\mu g\, g^{-1}$ for Zn (mean = 35.0 $\mu g\, g^{-1}$) in the wheat grown in Mexico. Sufficient genetic variation exits within the wheat germplasm to substantially increase Fe and Zn concentrations in wheat grain. There was a high correlation between grain-Fe and grain-Zn concentrations in the wheat lines studied. These findings indicated that it should be possible to improve Fe and Zn levels in wheat grain simultaneously through plant breeding. Many studies exploring required variability for grain iron and zinc content in landraces, wild and related germplasm, suggested that wild relatives had three to four times higher grain iron and zinc content than the modern hexaploid wheat cultivars, and the sitopsis section of *Aegilops* species possesses the highest variability for grain iron and zinc content (Chhuneja et al., 2006; Rawat et al., 2009).

Under the biofortification program, synthetic wheat was developed by crossing tetraploid emmer wheat with *T. taushii* accessions with high iron and zinc content (Monasterio and Graham, 2000; Calderini and Monasterio, 2003). In an attempt to improve nutritional qualities in wheat, Uauy et al. (2006) cloned a high grain protein (*Gpc-B1*) locus using wild wheat *T. turgidum* ssp. *dicoccoides* which induced early senescence and increased sequestration of iron and

zinc from leaves to grains. Exploiting useful variability from wild relatives is an uphill, difficult task due to sterility and reduced chromosome pairing in the interspecific hybrids. In the case of wheat, extensive back crossing is required to get BC_1 plants, which can be avoided by synthetic amphiploids (Rawat et al., 2009; Tiwari et al., 2009). It has been reported that chromosomes of group 2 and 7 of wild wheats and *Aegilops* species carry genes for high grain iron and zinc content. In a diploid wheat recombinant inbred lines (RIL) population, three quantitative trait loci (QTLs) have already been mapped for grain Fe and Zn content on chromosomes 2A and 7A (Tiwari et al., 2009, 2010). Peleg et al. (2009) also mapped three major QTLs for grain iron and zinc concentrations in a tetraploid wheat *T. durum–T. dicoccoides* RIL population, and the marker interval for a QTL common for zinc and iron on chromosome 7A was the same in both the above-cited studies. Shi et al. (2008) reported seven QTLs on chromosomes 1A, 2D, 3A, 4A, 4D, 5A, and 7A for zinc content in a double haploid wheat population of Hanxuan10 and Lumai14. Four zinc QTLs were also identified on chromosomes 3D, 4B, 6B, and 7A in a double haploid wheat population by Genc et al. (2009). Table 9.2 includes successful examples of mapping of some QTLs for high grain micronutrients in cereals.

TABLE 9.2 Tagging and Mapping of QTLs for High Grain Micronutrients Across Cereals

Cereal Crop	Micronutrient	Number of QTLs	Chromosome	Reference
Wheat	Zinc	11	4A, 4D, 2D and 3A	Shi et al (2008)
Wheat	Iron	2	2A and 7A	Tiwari et al (2009)
Wheat	Zinc	1	7A	Tiwari et al (2009)
Rice	Iron	3	2S, 8L, and 12L	Stangoulis et al (2007)
Rice	Zinc	2	1L and 2L	Stangoulis et al (2007)
Wheat	Iron	1	4	Peleg et al (2008)
Wheat	Iron and zinc	2	7	Peleg et al (2008)
Wheat	Zinc	4	3D, 4B, 6B, and 7A	Genc et al (2009)
Rice	Iron and zinc	14	1, 3, 5, 7 and 12	Anuradha et al (2012)
Wheat	Iron zinc and protein content	1	6B	Distelfeld et al (2007)
Wheat	Iron, zinc, copper, and manganese	1	5	Ozkan et al (2006)
Wheat	Iron and zinc	3	7A	Peleg et al (2009)

TABLE 9.3 The Mean and Range in Concentrations (Dry Weight Basis) of Fe and Zn in Six Sets of Brown Rice Germplasm (939 Genotypes) Grown under Similar Conditions at IRRI, Los Bãnos, Philippines

Genetic sets	Fe ($\mu g\,g^{-1}$)		Zn ($\mu g\,g^{-1}$)	
	Mean ± SE	Range	Mean ± SE	Range
Traditional and improved lines	13 ± 2.6	9.1–22.6	24.0 ± 4.7	13.5–41.6
IRRI breeding lines	10.7 ± 1.6	7.5–16.8	25.0 ± 7.6	15.9–58.4
Traditional and improved lines	12.9 ± 3.1	7.8–24.4	24.4 ± 4.7	16.5–37.7
Tropical japonicas	12.9 ± 1.5	8.7–16.5	26.3 ± 3.8	17.1–40.1
Popular lines and donors	13.0 ± 2.5	7.7–19.2	25.7 ± 4.6	15.3–37.3
Traditional and improved lines	13.8 ± 2.3	10.8–18.0	24.2 ± 4.1	19.9–33.3

SE, standard error of the mean.
Adapted from Graham et al. (1999).

Similarly for rice, researchers at IRRI have been evaluating the genetic variability of Fe concentration in rice grain since 1992. The range of Fe and Zn concentrations within the six sets of rice genotypes ($n = 939$) tested in a study was 7.5–24.4 $\mu g\,g^{-1}$ for Fe, and 13.5–58.4 $\mu g\,g^{-1}$ for Zn (Graham et al., 1999) (Table 9.3). Thus, within those genotypes tested, there was about a four-fold difference in Fe and Zn concentrations, suggesting some genetic potential to increase the concentrations of these micronutrients in rice grain.

Rice varieties Jalmagna, Zuchem, Xua Bue Nuo, Madhukar, IR64, and IR36 were found to have the highest iron and zinc contents. Moreover Jalmagna, which is a traditional variety of eastern India, shows twice the Fe and 40% more Zn content than IR36. Grain Fe and Zn content was found to be four times higher in some aromatic rice lines than in popular cultivars (Graham et al., 1999; Gregorio et al., 2000). These results indicate that there is significant genetic diversity in the rice genome also to allow substantial increase in Fe and Zn concentrations in rice grain. QTL mapping for high grain iron and zinc on various rice chromosomes is shown in Table 9.2.

Maize, which is a staple crop of southern and eastern Africa, is also low in Fe and Zn content (CIMMYT, 2000). However, genetic variability for Fe and Zn has been reported by Banziger and Long (2000) in white grained tropical maize germplasm: in the range 16.4–22.9 $\mu g\,g^{-1}$ for Fe and 14.7–24.0 $\mu g\,g^{-1}$ for Zn, respectively. They also evaluated 1814 accessions in 13 trials over 6 years and reported a range in grain of 9.6–63.2 mg-Fe/kg and 12.9–57.6 mg-Zn/kg. In many developing countries of Latin America, Africa, and Asia, maize is the major staple food and often the only source of protein. Screening for maize

lines with better amino acid profile led to discovery of *opaque 2* mutant maize in Connecticut, USA (Mertz et al., 1964) with high lysine and tryptophan content. Through interdisciplinary research efforts, scientists at International Maize and Wheat Improvement Center (CIMMYT), Mexico developed quality protein maize (Vasal, 1999). Several QPM varieties and hybrids have been released in the areas where maize is a staple food, and studies have shown a beneficial impact on growth (height and weight) in children.

2.2 Genetic Engineering Approaches

Genetic engineering approaches have been successfully applied for biofortification of a few traits in cereals (Naqvi et al., 2009; Wirth et al., 2009; Masuda et al., 2012). This has been tried by introduction of genes that code for micronutrient-binding proteins, overexpression of storage proteins already present, and/or increased expression of proteins that are responsible for micronutrient uptake into plants (Lonnerdal, 2003). The bioavailability of the existing or biofortified micronutrients can be also enhanced through reduction of anti-nutritional factors or increasing the amount of enhancers in the plant products through genetic engineering of the requisite pathways or using the same during processing.

Two novel iron-binding proteins have been incorporated into rice (Suzuki et al., 2003; Nandi et al., 2002; Goto et al., 1999). Suzuki et al. (2003) reported very high expression level (5 g of lactoferrin/kg of grain; 6% of total protein) of human lactoferrin, the major iron-binding protein in breast milk, in rice. Each molecule of lactoferrin binds two atoms of ferric iron, and the recombinant lactoferrin was shown to be fully iron saturated. Similarly, Goto et al. (1999) reported the insertion of soybean ferritin gene in rice, and the iron content in a few transformants was found to be two- to three-fold higher than that of wild-type. Another research group, by using *Agrobacterium*-mediated transformation and the rice glutelin promoter, transformed the ferritin gene from *Phaseolus vulgaris* into rice (Lucca et al., 2001). They were able to double the iron content of dehusked rice. Recently, Wirth et al. (2009) reported two rice genes playing key roles in mobilization and storage of iron. One gene encodes nicotianamine synthase, the enzyme that produces nicotianamine. Nicotianamine chelates iron temporarily and facilitates its transport in the plant. The second gene encodes the protein ferritin, which comprises a sink for iron in the center of the endosperm, since the ferritin gene was expressed under the control of an endosperm-specific promoter. Masuda et al. (2012), by using three transgenic approaches involving iron storage protein ferritin with endosperm specific promoter, overproduction of metal chelator nicotianamine and an iron (II) nicotianamine transporter *OsYSL2* under sucrose transporter promoter, enhanced iron concentration to 4.4-fold higher in transgenic polished rice than the non-transgenic seeds.

Genetic engineering also offers an effective way to increase the vitamin content of staple crops. Naqvi et al. (2009) developed inbred South African

transgenic maize plants in which the levels of three vitamins were increased specifically in the endosperm through the simultaneous modification of three separate metabolic pathways. The transgenic kernels contained 169-fold the normal amount of β-carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate. In the case of rice, the removal of aleurone layers during processing removes most of the oil-rich compounds, including provitamin A. The endosperm is devoid of vitamin A, thus people depending solely on rice as staple crop suffer from vitamin A deficiency. Burkhardt et al. (1997) first identified the C20 precursor molecule geranylgeranyl diphosphate in the wild type rice endosperm and transferred the phytoene synthase (*PSY*) gene from daffodils (*Narcissus pseudonarcissus*) into japonica rice variety Taipei 309. The transgenic phytoene synthase could condense geranylgeranyl diphosphate to phytoene, but carotenoid compounds could not be obtained. Ye et al. (2000) cloned the carotene desaturase gene (*CRT1*) from *Erwinia uredonora* along with the *PSY* gene. The *CRT1* gene catalyzed the conversion of phytoene to lycopene. Further cloning of lycopene β-cyclase (*β LCY*) DNA proved to be dispensable as rice transgenic plants with a two-gene (*PSY* and *CRT1*) and with a three-gene construct (*PSY*, *CRT1*, and *β LCY*) produced the same amount of carotenes. These lines became the prototype for lines for golden rice 1 production and accumulated up to $1.6\,\mu g\,g^{-1}$ of carotenoids in the endosperm (Al-Babili and Beyer, 2005). Soon after Paine et al. (2005) could identify the rate-limiting nature of the phytoene synthase catalysis step by cloning *PSY* genes from maize. The carotenoid synthase could be increased to $37\,\mu g\,g^{-1}$ when maize *PSY* genes were used. These experiments led to development of golden rice 2.

2.3 Physiological and Molecular Basis for Micronutrient Accumulation in Grains

For increased micronutrient metal uptake by roots, the levels of the available micronutrients in the root–soil interface must be increased to allow for more absorption by root cells. It could be achieved by stimulating certain root cell processes that alter micronutrient solubility and movement to root surfaces, such as the rate of root cell efflux of H and metal-chelating compounds and reductants. The Strategy-I dicotyledonous plants influx protons into the rhizosphere, followed by reduction of Fe^{3+} to usable Fe^{2+} form through the activity of ferric chelate reductases, such as FRO2. FRO2 is expressed specifically in roots and, along with other membrane-bound proteins including FRO6, FRO7, and FRO8b, takes part in iron transport in shoots (Mukherjee et al., 2006; Palmer and Geurinot, 2009). In the next step, specific iron-regulated transporter (IRT) proteins transport the readily soluble Fe^{2+} ions and Zn, Mn, and Cd into the plant roots (Rogers et al., 2000; Takahashi et al., 2011).

Members of the Poaceae family come under Strategy-II plants. Cultivation of rice, wheat, maize, and other cereals in conditions of limiting mineral availability (calcareous and saline sodic soil which contains iron in highly non-available form, i.e. Fe^{3+}) leads to lower iron uptake. Under these conditions, roots of various graminaceous plants secrete mugineic acids (MAs) called phytosiderophores (PS) into the rhizosphere, which chelate metals and are then absorbed by the plant as metal-chelator complexes (Mori, 1999; Takagi et al., 1998). Significant qualitative and quantitative differences for the production of mugineic acids have been observed among cereals. Among cereals, barley has been extensively explored for studying mineral uptake using phytosiderophores. Barley, as a Strategy-II plant, secretes mainly four types of mugineic acid family derivatives: mugineic acid (MA), 2′-deoxymugineic acid (DMA), 3-epihydroxymugineic acid (epi-HMA), and 3-epihydroxy-2-hydroxy mugineic acid (epi-HDMA) (Mori, 1999). The different forms of mugineic acid act as enhancers for the uptake of iron and zinc, and this provides tolerance to barley plants against low iron availability. Rice, wheat, and maize secrete only DMA in relatively low amounts, which makes them sensitive to low iron availability (Mori et al., 1990). Biosynthesis of MAs begins with S-adenosylmethionine, which is the precursor for the pathway. The biosynthetic pathway for mugineic acids has been studied extensively, and all the genes involved in this system have been cloned and characterized (Okumura et al., 1994; Takahashi et al., 1999; Bashir et al., 2006). Nozoye et al. (2011) reported that in barley and rice the efflux of deoxymugineic acid under stress conditions involves the *TOM1* and *HvTOM1* genes. In the next step the chelated metal-phytosiderophore complexes are taken up by an integral membrane protein of roots and shoots called Yellow Stripe 1 (YS1) transporter (Curie et al., 2001). Araki et al. (2011) reported HvYSL2, a metal–PS transporter that preferentially transports Fe (III)–PS. It plays a unique role in delivering essential metals in barley (Araki et al., 2011). Lee et al. (2012) reported a two-fold increase in iron content in seeds by over-expressing *OsNAS2* whereas Ishimaru et al., (2010) over-expressed *OsYSL2* using phloem specific *OsSUT1* promoter (phloem loading) and reported a four-fold increase in iron content of polished rice. These studies suggested that metal transport and sequestration from soil can be increased by coupling increased synthesis and release of phytosiderophores along with increased expression of genes encoding YSL proteins (Takahashi et al., 2001; Ishimaru et al., 2010; Lee et al., 2012). Screening of wild germplasm under iron sufficient and deficient conditions suggested 3–4 fold higher release of MA in some of the sitopsis sections of *Aegilops* species, including *Aegilops kotschyi* and *Ae. longissima* (Neelam et al., 2011). It would be interesting to see whether these wild sitopsis species are releasing only DMA or they are releasing all forms of MAs. Neelam et al. (2012) reported that group 2 wheat-*Aegilops* addition lines released higher MA in the rhizosphere under sufficient and deficient growth conditions which was positively correlated with high grain iron and zinc concentrations.

2.4 Sequestration of Mineral in Endosperm

Most of the mineral content present in the grains is confined to the aleurone layer and embryo, which is removed during milling and processing. A useful approach towards increasing the iron and zinc status of cereal-based diets would be to increase the sequestration of minerals to the endosperm, but little is known about transporters facilitating distribution of minerals across the grains (Mori, 1999; Curie et al., 2009; Palmer and Geurinot. 2009; Conn and Gilliham, 2010). Recently, with the development of techniques such as laser capture microdissection, it has been possible to study the expression of different genes across different tissues of grains (Borg et al., 2009; Tauris et al., 2009; Schiebold et al., 2011).

Transfer cells are the interface between the phloem of the maternal tissue and the endosperm tissue and have numerous wall ingrowths increasing the surface area by more than 20-fold. Highly expressed in transfer cells in barley were: heavy metal ATPases (HMA), zinc-regulated iron-regulated protein (ZIP), cation diffusion facilitator (CDF), natural resistance associated macrophage proteins (Nramp), vacuolar iron transporter 1 (VIT1), cation exchanger (CAX), yellow stripe like (YSL), metallothionins (MT), nicotinamine synthase (NAS), and nicotinamine amino transferase (NAAT) (Tauris et al., 2009). It has been proposed that Zn combines with NA or MA and then is taken up from the phloem and stored in vacuoles by CDF, VIT1, ZIP1, and CAX family transporters. YSL and ZIP transporters have been proposed to capture zinc–NA complexes from flowing back to the apoplast. The aleurone and embryo expression profiles were similar. HMA8, ZIP, and CDF were expressed in both the tissues at about the same level, although Nramp3, ZIP1, CAX1a, VIT1, NAS9, and NAATB were expressed at a higher rate in aleurone than in embryo. Nramp3 has been proposed to mediate efflux of Zn from the aleurone cells, while others control movement to the aleurone cells where the Zn is stored chiefly in vacuoles. The expression of transporter genes in the endosperm tissue was limited. Ramesh et al. (2004) over-expressed Zn transporter *AtZIP1* of *Arabidopsis* in barley under an ubiquitin promoter. The transgenic lines produced smaller seeds with high Zn concentration. Manipulating the expression of genes regulating CAX transporters has been proposed as an approach to increase Zn concentrations in the edible tissues of transgenic plants (Shigaki et al., 2005).

2.5 Bioavailability of Micronutrients

Bioavailability is the absorption and utilization of nutrients by human beings. Some common inhibitors in staple crops such as phytic acid, fibers, lignins, tannins, oxalic acid, and lectins (Graham et al., 2001) inhibit bioavailability. There are also some promoters, such as ascorbic acid, citric acid, fumaric acid, sulfur-containing amino acids, long-chain fatty acids, and selenium, which

facilitate rapid uptake of micronutrients by the intestinal cells. Phytic acid is myo-inositol hexakisphosphate, the negative charges of which chelates divalent minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} strongly. Phytate constitutes 1–3% of seed weight and accounts for 60–90% of total phosphorus in seeds (Graf, 1983). During germination, the enzyme phytase is activated to release phosphates from phytate. Bioavailability can be enhanced by lowering phytic acid in low-phytic-acid mutants of food crops and by transgenic expression of phytic acid-degrading enzyme, phytase, in the seeds.

2.5.1 Lowering Phytic Acid in Grains and Products

Phytic acid synthesis in plants starts from conversion of glucose 6-phosphate to inositol-3-phosphate by myo-inositol-3-Pi synthase (MIPS) followed by inositol phosphate kinases. Therefore mutations in genes encoding MIPS and inositol polyphosphate kinases are the targets for production of low-phytic-acid (*lpa*) mutants. The seeds of *lpa* mutants have been found to be viable and normal. Low-phytic-acid mutants are being developed either by chemical- or radiation-induced mutagenesis. These *lpa* mutants include *lpa1* mutant of maize (Raboy et al., 2000), barley (Larson et al., 1998; Rasmussen and Hatzack, 1998), and rice (Larson et al., 2000). Breeding programs for low phytic acid and high inorganic phosphate are associated with some undesirable characteristics of nutritional quality, disease susceptibility, and yield, due to inhibition of inositol metabolism in the vegetative tissue. Thus the selection of seed-specific *lpa* mutants which have normal vegetative phytic acid content can be more useful in the breeding programs. The maize *lpa3* mutant and the barley *lpa1* mutant are embryo and aleurone specific, respectively, without having any deleterious effect on the other characteristics, and have a larger amount of free phosphate. Antisense technology has been employed for deriving seed-specific *lpa* mutants. Shi et al. (2007) showed that maize *lpa1* mutants are defective in a multidrug-resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter that is highly expressed in embryos, and within immature endosperm, germinating seed, and vegetative tissues. Silencing the gene in an embryo-specific manner in normal maize resulted in low phytic acid and high Pi (inorganic phosphate) content without any significant alteration in seed dry weight. In rice, attempts have been made to generate low-phytic-acid transgenics by silencing the rice Ins (3) P (1) synthase gene, *RINO1*. Kuwano et al. (2006), working in the University of Tokyo, for the first time could produce a transgenic rice line with low phytic acid and high inorganic phosphate level by silencing the *RINO1* gene under the control of the rice major storage protein glutelin GluB-1 promoter. In another study, Kuwano et al. (2009) reported production of transgenic rice by silencing the *RINO1* with 68% less seed phytic acid, having no negative effect on seed weight, germination, or plant growth.

Phytases, both microbial and plant sources, are the enzymes for sequential removal of phosphates from the phytic acid molecule. Action of phytase thus makes most of the micronutrients available for absorption by the intestinal cells.

Two categories of phytases have been identified by the International Union of Biochemistry: 3′ phytases that initiate the removal of phosphate from the 3′ position, and 6′ phytases that act on the 6′ position. The former type is predominant in microbes and the latter in plants. Many approaches have been employed for lowering levels of phytic acid in food. These methods include soaking, cooking, germination, and fermentation. Fermentation covers both microbial and enzymatic processing. These methods of food processing are not fully helpful, as phytate is not fully hydrolyzed by the phytases occurring naturally in plants and microorganisms. Addition of phytase preparations during food production is an alternative to optimize phytate dephosphorylation. The added phytases must be thermotolerant and show wide pH range variation in activity to be applied in food processing. Microbial phytases are highly thermophilic with a wide range of pH. *Aspergillus niger*, *A. fumigatus*, and *Rhizopus oligosporus* are the major source of industrial phytases (Greiner and Konietzny, 2006). A transgenic approach has been employed to transfer thermophilic microbial phytases to cereals to enhance bioavailability of micronutrients. Many model and crop plants, including tobacco, canola, soybean, wheat, rice alfalfa, *Arabidopsis*, maize, and others have been successfully transformed for microbial phytase production (Gontia et al., 2012). Lucca et al. (2001) achieved high expression of a heat stable fungal (*Aspergillus fumigatus*) phytase into rice, which was expected to remain active after food processing such as cooking; however, to the contrary, the *in-planta*-produced phytase showed decreased enzymatic activity during cooking. The *phy A* gene from *A. niger* has also been introduced into wheat by Brinch-Pederson et al. (2000, 2007). Holme et al. (2012) have reported cisgenic barley with improved phytase activity. Various approaches for higher expression of microbial phytases in transgenic plants, such as codon usage modifications, use of secretory signals, and glucosylation, and their localization to target regions, are being used (Gontia et al., 2012).

2.5.2 Animal Feed Trials and Cell Line Studies

The phytic acid in seeds chelates substantial amounts of phosphate, calcium, iron, and zinc, and hence the feeds for monogastric animals have to be fortified for production of healthy animals incurring higher costs and eutrophication. Ertl et al. (1998) first used maize *lpa1* mutant as a chick feed and found 46% and 49% greater blood phosphate and calcium level, respectively, than for chicks fed with wild maize. Transgenic plants over-expressing phytases have also been tried in various feed trials, showing enhanced absorption of various minerals by lowering the phytic acid content (Gontia et al., 2012). Han et al. (1994), using the Caco-*2* cell assay, reported the inhibitory effect of phytic acid on iron and zinc absorption. A 50% increase in iron absorption by Caco-*2* cells from *lpa1-1* maize was reported as compared with wild type maize (Raboy, 2007). Recently, Salunke et al. (2011, 2012), using Caco-*2* cell lines, found negative correlation of iron and zinc uptake from high iron and

zinc wheat derivatives with phytic acid content. Iron absorption was found to be 49% greater in men when fed with tortillas from *lpa 1-1* maize over the wild type maize (Mendoza et al., 1998). Adams et al. (2002) found 30% zinc absorption when healthy adults were fed with *lpa1-1* mutant as compared with 17% for wild type maize. These studies indicate that the lowering of phytic acid in the diet enhances micronutrient availability. Thus low-phytic-acid crops and phytase over-producing transgenic plants can be very helpful in combating micronutrient malnutrition in both traditional and biofortified crops, provided that the plant growth associated disadvantages can be addressed.

3. MICRONUTRIENT CONCENTRATION AND GRAIN YIELD

A number of studies have been carried out to find the effect of increased grain minerals content on grain yield (Graham et al., 1999). The wild relatives of wheat and land races have smaller seeds than modern wheat cultivars and higher concentration of minerals in grain. Modern elite wheat cultivars with larger seeds and higher starch content in grains have lower mineral concentration, which might be due to the dilution effect of a certain amount of micronutrients taken up for grain sequestration. The Zn concentration was found to be negatively correlated with grain yield in many field trials involving bread and durum wheat cultivars, whereas no significant correlation was observed between increased Fe concentration and grain yield (Oury et al., 2006; Zhao et al., 2009; Ficco et al., 2009). Welch and Graham (2004) reported no significant linkage between increased grain Fe and Zn concentration and grain yield. Many other reports have shown a consistently positive association between high grain protein content and Fe and Zn concentration (Distelfeld et al., 2007). Similarly, using two diverse populations of pearl millet, Gupta et al. (2009) reported no significant penalty of enhanced grain Fe and Zn concentration on grain yield and 1000 grain weight. In several studies among cereals the higher protein content has been reported to be negatively correlated with yield, whereas Regvar et al. (2011) found the protein globoids in the aleurone layer of wheat to be the preferential storage site for various micronutrients, suggesting that the higher micronutrient content may lead to yield penalty.

4. CONCLUSION

The global food system is failing to deliver adequate quantities of healthy, nutritionally balanced food, especially to resource-poor underprivileged people, leading to micronutrient malnutrition. The malnutrition of minerals (Fe, Zn) and vitamin A is a major food-related primary health problem among populations of the developing world where there is a heavy dependence on cereal-based diets and limited access to meat, fruits, and vegetables. Hence, in addition to the traditional objectives of disease resistance, yield, drought tolerance, etc., plant breeders have to exploit biofortification as a key objective for

the enrichment of staple crops, as this approach offers the best possible and economical solution for eradication of micronutrient malnutrition or hidden hunger. Existing variability in staple crops' germplasm offers enormous possibilities of biofortification in these crops through conventional and molecular breeding approaches. Genetic engineering is also another obvious alternative to enhance the micronutrient levels in staple crop plants. Enhanced bioavailability of the micronutrients in traditional as well as biofortified crops is equally important. Even once biofortification has been shown to be efficacious, successful introduction of biofortified foods will still require the addressing of various socioeconomic and sociopolitical challenges, to popularize their cultivation by farmers and ultimately to gain consumer acceptance for biofortified crops, thereby increasing the intake of the target nutrients. A multi-tier coordinated strategy will play a pivotal role in overcoming hidden hunger.

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