**Combating Mineral Malnutrition through Iron and Zinc Biofortification of Cereals**

Zaigham Shahzad, Hatem Rouached, and Allah Rakha

**Abstract:** Iron and zinc are 2 important nutrients in the human diet. Their deficiencies in humans lead to a variety of health-related problems. Iron and zinc biofortification of cereals is considered a cost-effective solution to overcome the malnutrition of these minerals. Biofortification aims at either increasing accumulation of these minerals in edible parts, endosperm, or to increase their bioavailability. Iron and zinc fertilization management positively influence their accumulation in cereal grains. Regarding genetic strategies, quantitative genetic studies show the existence of ample variation for iron and zinc accumulation as well as inhibitors or promoters of their bioavailability in cereal grains. However, the genes underlying this variation have rarely been identified and never used in breeding programs. Genetically modified cereals developed by modulation of genes involved in iron and zinc homeostasis, or genes influencing bioavailability, have shown promising results. However, iron and zinc concentration were quantified in the whole grains during most of the studies, whereas a significant proportion of them is lost during milling. This makes it difficult to realistically assess the effectiveness of the different strategies. Moreover, modifications in the accumulation of toxic elements, like cadmium and arsenic, that are of concern for food safety are rarely determined. Trials in living organisms with iron- and zinc-biofortified cereals also remain to be undertaken. This review focuses on the common challenges and their possible solutions related to agronomic as well as genetic iron and zinc biofortification of cereals.

**Keywords:** Biofortification, bioavailability, Iron, zinc, biotechnology

**Introduction**

Iron and zinc deficiencies are widespread health problems. Iron deficiency is the most common nutritional disorder in the world, and almost 1.6 billion people are suffering from iron deficiency (De Benoist and others 2008). Iron deficiency anemia is by far the most widespread micronutrient deficiency, and it results in impaired physical growth, mental development, and learning capacity (Bouis 2003). Zinc deficiency is equally serious and is ranked as the 5th leading risk factor for diseases in the developing world (Maret and Sandstead 2006). Numerous health problems link zinc deficiency to retarded growth, skeletal abnormalities, delayed wound healing, increased abortion risk, and diarrhea (Salgueiro and others 2000). Approximately one-third of the world’s population is suffering from zinc deficiency (Hotz and Brown 2004). The situation is even more adverse in developing countries where more than half of the children and pregnant women are suffering from iron and zinc deficiencies (Seshadri 1997, 2001; Caulfield and others 1999). This situation is largely attributed to the high consumption of cereal based foods, rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.), in these countries (Pfeiffer and McClafferty 2007). Edible parts (endosperms) of modern cereal cultivars are inherently poor in iron and zinc. Iron and zinc concentration in whole grain of wheat are in the range of 29 to 73 mg/kg and 7 to 85 mg/kg, respectively (Rengel and others 1999; Cakmak and others 2004). However, more than 75% of these nutrients is located in the seedy parts other than endosperm which is lost during milling (Slavin and others 2000; Ozturk and others 2006). The concentration of iron in the brown rice ranges from 6.3 to 24.4 mg/kg and zinc concentration ranges from 15.3 to 58.4 mg/kg (Gregorio and others 2000). However, polished rice, the principal form of rice consumed, on an average contains only 2 mg/kg iron and 12 mg/kg zinc (Barry 2006).

Apart from the cereals inherent inability to accumulate high iron and zinc, one major reason for their low accumulation in edible parts is the cultivation of cereals on zinc-deficient soils, particularly in developing countries like Pakistan, China, India, Iran, and Turkey (Cakmak and others 1999; Alloway 2009). For instance, in Turkey, zinc concentration in wheat grains grown on zinc-sufficient soils ranged between 20 and 30 mg/kg, whereas on the zinc-deficient soils this range fell enormously to 5 to 12 mg/kg (Kalayci and others 1999; Erdal and others 2002). The Green Revolution is also considered to have contributed to the prevalence of these micronutrient deficiencies in soils because it promoted the use of high-yielding varieties, large-scale irrigation, and macronutrient fertilizers (nitrogen, phosphorus, potassium;
Biswas and Benbi 1997; Welch and Graham 1999; Dar 2004). It is considered that high yielding varieties led to the dilution effect of micronutrients due to increased 1000 kernel weights. The existence of a negative relationship between irrigation and iron and zinc uptake (Scagel and others 2012) and a similar negative relationship between phosphorus and iron and zinc uptake (Saha and others 2013) also lead to lower the accumulation of these micronutrients in the cereal grains. Since the edible parts of the cereals are poor in iron and zinc, thus heavy dependence of people from developing countries on these foods results in the development of large-scale iron and zinc malnutrition. To alleviate iron and zinc deficiency, it is required to increase iron and zinc concentration in the endosperm to 8 and 30 mg/kg, respectively (www.harvestplus.org). Currently, there is growing concern to address micronutrient malnutrition through different interventions. Typically, these interventions are categorized into 4 major groups: pharmaceutical supplementation, industrial fortification, dietary diversification, and biofortification (Tontsirin and others 2002; Meenaski and others 2007).

Iron and zinc pharmaceutical supplementation and industrial fortification are not considered cost-effective and only very few governments have the resources to finance such kinds of expensive interventions (Meenaski and others 2007; Stein 2007). Moreover, several other issues such as negative interaction between iron and zinc bioavailability (Wasantwisut and others 2006), frequency of supplementation, selection of food products to be fortified, impact of fortificants on taste, texture, and appearance of food, and availability of fortificant (Gibson and Ferguson 1998) need to be carefully addressed before undertaking these interventions. Dietary diversification offers greater prospects to overcome micronutrient malnutrition (Gibson and Hotz 2001; Johns and Eyzaguirre 2007). For instance, in India, Bangladesh, and Tanzania, small-scale kitchen-gardening projects have shown promising results by diversifying cultivation and consumption of high β-carotene fruits and vegetables (Frison and others 2006). However, to achieve success on a large scale, poverty alleviation and change in food habits of high-risk groups, the rural poor, children under 5, and child bearing women, are prerequisites. Bouis (2003), based on surveys in Bangladesh and the Philippines, showed that rich families already consume a diversified diet, whereas poor families do not have enough resources to purchase a diversified diet. Thus, considering the limitations linked to the above-mentioned 3 types of interventions to alleviate iron and zinc deficiencies, the biofortification of staple food crops is considered the most viable option to help alleviate iron and zinc malnutrition.

We will discuss the opportunities and challenges associated with iron and zinc biofortification of cereals in this review. Iron and zinc biofortification of cereals aims at either increasing the accumulation of these minerals in edible parts or increasing their bioavailability (Figure 1). Currently, 2 research strategies to achieve this are mineral fertilization and crop biofortification. Mineral fertilization can mainly increase the accumulation of iron and zinc in edible parts, whereas crop biofortification has the potential to fulfill both aims. Both these strategies have their own advantages and limitations that will be discussed below.

Minerals Fertilization

Iron fertilization

Soil iron fertilization is believed to have little or no effect on iron concentration of grains (Narwal and others 2010). Thus much of the work has been focused to identify the effects of foliar application on iron accumulation in grains. As shown in Table 1 foliar application is reported to increase iron concentration by 20% to 70% in the grains of bread wheat (Shukla and Warsi 2000; Habib 2009; Zeidan and others 2010; Zhang and others 2010). Recently, effects of foliar application of different forms of iron fertilizer at different plant developmental stages were studied in rice and it was shown that application of the DTPA-Fe form at the anthesis stage resulted in about 20% increase in iron content of polished rice grains (He and others 2013). In addition to grain iron concentration, iron fertilization positively influences the grain zinc concentration in rice and wheat (Shukla and Warsi 2000; Fang and others 2008; Habib 2009; Shi and others 2010; Zeidan and others 2010).

Zinc fertilization

It is generally found that zinc deficiency in human beings is associated with zinc-deficient soils (Cakmak and others 1999; Al-loway 2009). This led to numerous studies to identify the effect of soil or foliar zinc fertilization on grain zinc concentration under varied agro-ecological conditions. Soil application of zinc resulted in 20% to 90% and 60% to 250% increase in grain zinc concentration in bread wheat (Triticum aestivum L.) and durum wheat (Triticum durum L.), respectively (Table 1; Cakmak and others 1997; Yilmaz and others 1997; Shukla and Warsi 2000; Yakan and others 2001; Shivay and others 2008; Cakmak 2010; Zhao and others 2011; Stomph and others 2011). As shown in Table 1 foliar application of zinc resulted in even a higher increase in grain zinc concentration than soil application in both bread and durum wheat (Cakmak and others 1997; Yilmaz and others 1997; Habib 2009; Zeidan and others 2010; Zhang and others 2010; Khoshogfarmanesh and others 2013). Studies of natural variation revealed the existence of notable differences for zinc accumulation in wheat grains between different wheat cultivars in response to soil and foliar application of zinc (Cakmak and others 1997; Yilmaz and others 1997; Khoshogfarmanesh and others 2013). Moreover, the timing of foliar application of zinc was also found to be crucial in determining the wheat grain zinc contents. Foliar application of zinc around flowering time was shown to produce the highest increase in zinc contents in the endosperm of wheat grains (Cakmak and others 2010). These findings suggest that both the zinc uptake and the remobilization are important factors that determine the zinc concentration in wheat grains, and natural variation for response to zinc fertilization is present in this species. Phloem is the only vascular tissue to reach the developing wheat grains, therefore zinc has to leave the xylem at some stage and become actively loaded into the phloem to reach the grains (Patrick and Offer 2001). The genetic and molecular mechanisms controlling this re-translocation from leaves to grains are still unknown, now considered a bottleneck to explain the natural variation of response to zinc fertilization between wheat cultivars, and to successfully exploit the potential of this mechanism to zinc biofortify wheat. In rice, the impact of soil or foliar application of zinc on increasing grain zinc concentration is not as strong as in wheat; generally, an increase in the range of 10% to 60% is found in grain zinc concentration in response to zinc fertilization (Table 1; Shivay and others 2007; Fang and others 2008; Phattarakul and others 2009). Similarly, very low increase in grain zinc concentration (up to 40%) was observed after soil application of zinc fertilizer in maize (Kanwal and others 2010). Foliar application of zinc, besides influencing grain zinc concentration, could also increase iron concentration in wheat, rice, and maize grains (Fang and others 2008; Habib 2009; Aref 2010; Zeidan and others 2010), and reduce cadmium toxicity and accumulation in cereals grown.
### Table 1–The effect of iron or zinc fertilization on accumulation of these minerals in cereal grains.

<table>
<thead>
<tr>
<th>Species</th>
<th>Variety</th>
<th>Nutrient method</th>
<th>Nutrient added</th>
<th>Percent increase in grain zinc (mg/kg)</th>
<th>Percent increase in grain iron (mg/kg)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Gerek-79</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>90</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yilmaz and others 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foliar</td>
<td>0.4% Zn</td>
<td>330</td>
<td>70</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dagdas-94</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>70</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bezostaja-1</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>70</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foliar</td>
<td>0.4% Zn</td>
<td>310</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td><em>Triticum durum</em> Desf.</td>
<td>Kunduru-1149</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>190</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foliar</td>
<td>0.4% Zn</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Bezostaja-1</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>60</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cakmak and others 1997</td>
</tr>
<tr>
<td></td>
<td>Atay-85</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Kunduru-1149</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>60</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-1252</td>
<td>Soil</td>
<td>23 kg/ha Zn</td>
<td>70</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Soil</td>
<td>5.2 kg/ha Zn</td>
<td>30</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Shukla and Warsi 2000</td>
</tr>
<tr>
<td></td>
<td>Balcali</td>
<td>Soil</td>
<td>21 kg/ha Zn</td>
<td>250</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cakmak 2010</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Zhengmai 9023</td>
<td>Soil</td>
<td>45 kg/ha Zn</td>
<td>30</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Hao and others 2011</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>SAMNYT-16</td>
<td>Soil</td>
<td>5 mg Zn kg&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>10</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Kunduru-1149</td>
<td>Soil</td>
<td>150 g/ha Fe</td>
<td>420</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-1252</td>
<td>Soil</td>
<td>150 g/ha Fe</td>
<td>90</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>G-30</td>
<td>Soil</td>
<td>0.5% Zn</td>
<td>210</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Zeidan and others 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foliar</td>
<td>1% Fe</td>
<td>20</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Jing 411</td>
<td>Foliar</td>
<td>1.8 kg/ha Fe</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foliar</td>
<td>1.8 kg/ha Fe and Zn</td>
<td>10</td>
<td></td>
<td>Zhang and others 2010</td>
</tr>
<tr>
<td><em>Oryza sativa</em> L.</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Soil</td>
<td>5.2 kg/ha Zn</td>
<td>50</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Shivay and others 2007</td>
</tr>
<tr>
<td><em>Oryza sativa</em> L.</td>
<td>Wuyunjing 7</td>
<td>Foliar</td>
<td>0.9 kg/ha Zn</td>
<td>20</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fang and others 2008</td>
</tr>
<tr>
<td><em>Oryza sativa</em> L.</td>
<td>TDK 7</td>
<td>Foliar</td>
<td>1.8 kg/ha Fe</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>Phattarakul and others 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foliar</td>
<td>1.8 kg/ha Fe and Zn</td>
<td>30</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Zea mays</em> L.</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Soil</td>
<td>50 kg/ha Zn</td>
<td>10</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Kanwal and others 2010</td>
</tr>
<tr>
<td></td>
<td>FHY-421</td>
<td>Soil</td>
<td>54 kg/ha Zn</td>
<td>40</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>Zea mays</em> L.</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Soil</td>
<td>24 kg/ha Zn</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
<td>Aref 2010</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND for no data were available.
<sup>b</sup> NI for no increase in grain iron or zinc concentration in response to iron or zinc fertilization.
Fe and Zn biofortification of cereals . . .

Figure 1—Cereals biofortification strategies. The strategies aiming to increase the accumulation of iron and zinc in endosperm are mentioned in yellow shapes and the strategies aiming at increasing the bioavailability are in green shapes. The impact that a strategy is expected to have for developing biofortified cereals is represented by the thickness of arrows, for example, increasing iron and zinc concentrations in cereal grains using genetic strategies is expected to have higher chances of success for producing iron- and zinc-biofortified cereals as compared to other strategies. The potential risks associated with different strategies are indicated by light blue shapes.

on cadmium-contaminated sites (Jiao and others 2004; Kölesi and others 2004). These findings strongly support the utilization of zinc fertilizer in cereal cultivation to produce zinc/iron-enriched cereals, but the success may depend upon the cultivar being used and agro-ecological conditions.

An additional benefit of using iron and/or zinc fertilizers in cereal production is their positive effect on yields. Numerous studies have established the positive relationship between provision of trace minerals and crop growth, thus they are recognized as essential when aiming for better grain yields (White and Zasoski 1999; Sotomayor-Ramirez and others 2003; Fageria and Bresheghello 2004; Rashid and Ryan 2004; Welsh and Graham 2004; Gupta 2005; Narwal and others 2010; Torkashvand 2011; Xi-wen and others 2011; Khoshgoftarmanesh and others 2013). Moreover, micronutrient provision to crops might result in more vigorous seedlings, lower vulnerability to plant diseases, and possibly improved drought resistance (Frossard and others 2000; Welch and Graham 2002, 2004; Bouis 2003). However, micronutrient fertilizers may also contain elevated amounts of toxic metals such as cadmium and repeated uses of the fertilizers at high dose over a prolonged period may increase cadmium accumulation in plants (Huang and others 2003). Thus, a judicious approach is required during the formulation and utilization of these fertilizers in cereal production to avoid contamination by toxic metals like cadmium.

Cereals Genetic Improvement

The goal of iron and zinc genetic biofortification is to develop crops with increased bioavailable contents of these minerals in edible parts. In the following section of this review, attention will be focused on the strategies that can be devised to increase the bioavailable contents of iron and zinc in the edible parts of cereals and future research challenges in the field of genetic biofortification. Mainly, 2 strategies can be chosen to produce cereal crops with the ability to fight iron and zinc hidden hunger: (i) increasing iron and zinc bioavailability; and (ii) increasing concentration of iron and zinc in grains.

Different genetic approaches can be chosen to develop iron- and zinc-biofortified cereals (Figure 2). However, major attention to date has been focused on the development of genetically engineered cereal grains which have increased bioavailable contents of iron and/or zinc. Conversely, fewer studies have been targeted on exploiting other fields of genetics like quantitative genetics, marker-assisted breeding, and mutation breeding to identify the genetic determinants controlling iron and zinc accumulation in grains, and thereby to use the desirable genetic resources in breeding programs. To orient the readers hereafter we will classify the genetic approaches into breeding and genetic engineering approaches. Here, breeding approaches entail quantitative genetics, marker-assisted breeding, and mutation breeding to identify the genetic determinants controlling iron and zinc accumulation in grains, and thereby to use the desirable genetic resources in breeding programs. To orient the readers hereafter we will classify the genetic approaches into breeding and genetic engineering approaches.
for agronomically desirable traits. Underlying genes can be cloned through fine mapping or map-based cloning approaches. Yet in the absence of precise knowledge of causal genes, markers tightly linked to these genes can be developed and tested/used in marker-assisted breeding programs. Despite huge progress, marker-assisted selection (MAS) had only a small impact on plant breeding so far largely because of lack of good knowledge of the effects of genetic background, QTL × environment interactions and epistatic effects (Collard and Mackill 2008). There also exists a knowledge gap between molecular biologists and plant breeders, and the application gap between research laboratories and plant breeding institutes. Additionally, the high cost is associated with the traditional types of molecular markers. The cost factor connected with genotyping is considered to be lowered markedly as a result of development of high-throughput single nucleotide polymorphism (SNP) genotyping platform like the ones already developed in rice (Zhao and others 2011) and maize (McMullen and others 2009). Recently published wheat genome sequences (Brenchley and others 2012; Ling and others 2013) will help in developing high-throughput SNP genotyping platform in this species as well. These innovations will help the breeders to develop genome-wide selection strategies. Despite numerous limitations, progress has been made in this field of marker-assisted breeding, which supports the feasibility of undertaking such an approach for iron and zinc biofortification of cereals. An excellent example of successful application of MAS for plant nutrition-related traits comes from rice, where a major QTL of phosphorus use efficiency, Pup1, was successfully introgressed from a phosphorus use-efficient rice genotype into a phosphorus use-inefficient rice genotype using molecular markers tightly linked to the QTL (Chin and others 2011). The success of this study is largely considered due to the major effect of Pup1 QTL on the trait, and introgression of a minor or modest effect QTL is expected to be far more difficult, yet achievable.

Genetic engineering is an alternative to the time-consuming and relatively expensive molecular breeding approach targeting the manipulation of an organism’s nucleic acid, thus enabling scientists to introduce desired traits into an organism. The organism thus developed is known as a genetically modified organism (GMO). The development and use of GMOs has been in place since 1994 when a genetically modified rotting-resistant tomato, Flav Savr, developed by the introduction of an antisense gene interfering with the production of the enzyme polygalacturonase was approved by the U.S. Food and Drug Administration (FDA) (James and Krattiger 1996). Since then a huge amount of resources has been invested to develop GMO crop varieties for different traits because it is easier and more rapid to develop a GMO variety than a non-GMO variety. During the last decade, grain nutritional quality has also attracted the attention of scientists. Recently, such a GMO was developed in rice and was named Golden Rice. An entire β-carotene biosynthetic pathway using genes from different species was introduced into rice endosperm through Agrobacterium-mediated transformation (Ye and others 2000), hence endosperm of GMO rice became rich in provitamin-A. The applications of genetic engineering to develop GM cereal varieties with increased
bioavailable iron and zinc concentration in grains will be discussed below.

**Increasing Iron and Zinc Bioavailability**

The bioavailability of iron and zinc can be increased by reducing the concentration of inhibitors which hinder the human absorption of dietary iron and zinc or increasing the concentration of enhancers which favor iron and zinc absorption. Cereal foods are the most important part of both human and animal diets. They provide proteins, carbohydrates, fiber, vitamins, minerals, antioxidants, and phytochemicals. Yet, they contain antinutritional factors (ANFs) such as phytate, tannin, and certain insoluble fibers which interfere with the absorption of iron and zinc (Gillooly and others 1984; Hambridge and others 2010; Petry and others 2012). The ANFs contain negatively charged groups which results in the creation of mostly insoluble complexes with numerous divalent and trivalent cations, thus making them unable to be absorbed during intestinal digestion. In cereals, phytate being the most predominant form of ANFs (O’Dell and others 1972; Lestienne and others 2005) have been the subject of numerous studies. Phytic acid (PA, also known as myo-inositol hexaphosphate) is the primary storage form of phosphorus (P) in seeds, typically accounting for 60% to 90% of the total seed phosphorus and contributing as much as 1.5% to the seed dry weight (Bohn and others 2008). The negatively charged phosphates in PA strongly bind to metallic cations (such as K, Mg, Mn, Fe, Ca, and Zn) to form a mixed salt called phytin or phytate (Bohn and others 2008). Metal cation-phytate complexes can be formed in 2 ways: simple phytate-mineral complexes or fibre-phytate-mineral complexes. The stability and solubility of the metal cation-phytate complexes depend on the individual cation, the phytate-to-cation molar ratio, pH value, and presence of other compounds in the solution (Greiner and others 2006). Phytate is predominantly found in the protein bodies of embryo and aleurone layers (Steadman and others 2001), where there is a high deposition of minerals as well. The chelation of iron and zinc with PA has a strong negative effect on absorption of these minerals in humans and other monogastric animals that largely lack the phytase enzyme, which is required to degrade phytate.

Dephytination can be achieved by numerous ways. Different processing methods like mechanical, thermal, and bioprocessing techniques have been employed to degrade phytate (Sandberg 1991; Schlemmer and others 2009; Frontela and others 2011). The extent of dephytination was shown to vary depending upon the rigor and intensity of processing operations. Usually, phytate is quite stable to cooking for prolonged periods (Schlemmer and others 2009). Likewise, extrusion cooking seems to have little or negative impact on the iron bioavailability (Sandberg and others 1987; Hurrell and others 2002). The decreased bioavailability of minerals was attributed to the loss of phytase activity during extrusion cooking. Owing to water solubility, phytate level may decrease during soaking (Gustafsson and Sandberg 1995; Liang and others 2008; Afify and others 2011). Further reduction can be achieved by soaking at the optimal temperature (55°C) and pH (5.5) for phytases (Schlemmer and others 2009). Dephytination can also be realized by activation of either endogenous phytase or external addition of phytase (Sandberg 1991; Luo and others 2014). Endogenous phytase activity in cereals varies with the species in question (Schlemmer and others 2009). Among cereals, rye is said to contain high phytase activity (5000 to 7000 U/kg) followed by wheat (1200 to 3000 U/kg) and barley (1000 to 2300 U/kg) (Greiner and Konietzny 2006). Relatively lower phytase activity has been observed in oats (100 to 500 U/kg), rice (150 to 350 U/kg), and corn (70 to 150 U/kg) (Steiner and others 2007). Endogenous phytases are activated during proofing of dough in bread-making (Sandberg and Svanberg 1991; Schlemmer and others 2009). The germination/malting of cereals is another such technique, believed to be quite helpful in increasing the bioavailability of iron and zinc in cereals (Krishnan and others 2012). Germination of cereals results in the activation of endogenous phytase which triggers the hydrolysis of phytate in grains (Luo and others 2014). Exogenous phytase addition is also practiced to improve the mineral bioavailability (Sanz-Penella and others 2008), and dephytination by the addition of exogenous phytase in porridges of wheat, rice, maize, and oats has been shown to improve iron bioavailability in human subjects (Hurrell and others 2003). In an *in vitro* digestion study in a Caco-2 cell model, it was demonstrated that PA significantly reduces (about 86%) bioavailability of iron from infant cereal diets (Jin and others 2009), and dephytination markedly improved iron bioavailability from these diets (Frontela and others 2009). In addition to iron, dephytination can also improve zinc bioavailability. In another study, dephytination of soy formula also improved zinc bioavailability (Lönnérdal and others 1988). Thus, it is anticipated that reduction in dietary phytate content is likely to result in an improvement in iron and zinc absorption. Accordingly, for the past few years, it is being advocated that iron and zinc malnutrition could be combated by reducing seed PA. Different breeding ad genetic engineering approaches that can help to develop cereal varieties with low-phytate will be discussed here.

**Breeding Approaches to Reduce Phytate**

Exploring natural variation may help us identify novel genetic mechanisms to reduce seed phytate in cereals. Liu and others (2006) characterized 186 wheat genotypes comprising landraces and cultivars for seed phytate concentration and found about 30% variation. The quantitative genetics approach is relatively more developed in a model plant, *Arabidopsis thaliana*, where various QTL mapping studies have been performed. A recombinant inbred lines (RILs) population developed by crossing Ler and Cvi was characterized for variation in seed phytate concentration and thereby used for QTL mapping (Bentsink and others 2003). The Ler × Cvi RILs displayed more than 2-fold variation for phytate concentration. More than 50% of the RILs displayed positive or negative transgression suggesting that new allelic combinations generated by mixing genomes of the 2 individuals offer great potential to reduce seed phytate concentration. Further, the authors mapped QTLs controlling seed phytate and found that one of the QTLs located on top of the chromosome 03 accounted for more than 60% of the total variance. Recently, 3 additional *A. thaliana* RIL populations were used for mapping QTLs of seed phytate concentration (Ghandilyan and others 2009). Positive as well as negative transgression for seed phytate concentration was consistently observed in all the populations, and QTLs for seed phytate concentration in these populations were also mapped to the top of chromosome 3 that explained more than 30% of the total variance. Thus, top of chromosome 3 in *Arabidopsis* seems very crucial to determine the variation in seed phytate concentration, and high percentage of variance explained by the QTLs in studies conducted under variable environmental conditions suggests that the underlying gene could have major and stable influences on the trait. Unfortunately, the gene underlying this variation has not been fine-mapped and thus characterized at the molecular level. A similar study was undertaken in rice to map the QTLs of seed phytate concentration, and iron and zinc concentration in rice...
seeds using doubled haploid population (DH) of IR64 × Azucena (Stangoulis and others 2007). Interestingly, only positive transgression was observed for seed phytate concentration in this DH population in contrast to Arabidopsis thaliana RIL populations. In DH population of rice accessions, two QTLs, one on chromosome 05 and another on chromosome 12, respectively, explained 24% and 15% of the total variance. Again, it is unfortunate that fine-mapping was not performed to identify the underlying genes. However, it is considered to be encouraging as far as marker-assisted breeding is concerned. Markers tightly linked to these regions can be developed and tested in real breeding populations for their efficacy to select low-phytate lines.

Low-phytic acid (lpa) mutants have been isolated thorough mutagenesis in cereals such as wheat (Guttieri and others 2004), rice (Liu and others 2007), maize (Raboy and others 2000; Pilu and others 2003; Shi and others 2003), and barley (Rasmussen and Hatzack 1998). Genetic studies of these mutants revealed various underlying genes such as myo-inositol, takashi’s protein and inositol phosphatase, and multidrug resistance-associated proteins (MRP) (Shi and others 2003; Shi and others 2005; Shi and others 2007; Kim and Tai 2011). Unfortunately, these lpa mutations have pleiotropic effects. Maize lpa mutants exhibit 23% reduction in seed dry weight (Raboy and others 2000), accompanied by a reduction of around 30% in germination (Pilu and others 2003). lpa mutations have also been shown to badly affect the yield in wheat and rice where up to 25% reduction in yield was observed in mutants impaired in the biosynthesis of PA (Guttieri and others 2006; Zhao and others 2008). Reduction in PA content may also affect other components of the grain. The much lower activities of ADP glycogen phosphorylase (ADP-Pgase) and starch phosphorylase (SPase) in the grains were observed at the filling stage in rice lpa mutants, resulting in decreased grain weight, low grain starch accumulation, and poor plumpness (Chun and others 2008). In barley lpa mutants malting may be difficult, as the lpa trait was associated with substantial reductions in diastatic power, a measure of how much starch-converting enzyme any given malt contains (Bregitzer and Raboy 2006). Recently, an interaction between lpa mutations and anthocyanin accumulation and compartmentalization in the kernels of maize lpa mutants was suggested (Badone and others 2010), which could lead to changes in the attraction of pollinators, signaling with microbes, male fertility, antimicrobial activity, UV protection; and in general the protection from oxidative damage (Winkel-Shiley 2002). Phytate are degraded by endogenous phytases which release phosphate and other minerals during seed germination that supports the growth and development of the seedling (Centeno and others 2003; Hemalatha and others 2007). Thus, it is anticipated that breeding lpa crops using mutation breeding may seriously hamper seed germination and plant performance, which will ultimately affect yields.

Genetic Engineering Approaches to Reduce Phytate

Phytases, a special class of phosphatases, catalyze the sequential hydrolysis of PA to produce less phosphorylated myo-inositol derivatives and inorganic phosphate. Seed phytate concentration decreases by expressing heterologous phytases in cereals either under the control constitutive promoters or tissue-specific promoters. A thermo-stable phytase gene of Aspergillus fumigatus (phyA) was expressed in rice endosperm under the control of glutelin promoter which resulted in a 130-fold increase in phytase activity in transgenic seeds (Lucca and others 2001). This much phytase activity was considered to be sufficient to completely degrade phytic acid in a simulated digestion experiment. However, the results were not confirmed after boiling the rice for 20 min in water where only 8% of the total phytase activity was retained. Brinch-Pedersen and others (2000) transformed Aspergillus niger phytase encoding gene (phyA) in wheat using 2 expression cassettes. In the 1st, an α-amylase signal peptide sequence was inserted between the promoter and the phytase coding region (Ubi-SP-Phy), and in the 2nd no α-amylase signal peptide was inserted (Ubi-Phy). The Ubi-SP-Phy transgenic seeds exhibited up to a 400% increase of phytase activity, while up to 56% increase was found in Ubi-Phy plants. In maize, overexpression of Aspergillus niger phytase gene (phyA2) in seeds using a construct driven by the maize embryo-specific globulin-1 promoter resulted in about 5000% increase in phytase activity and 30% decrease in seed phytate concentration (Chen and others 2008). During most of these studies characterization of the transgenics for agronomic characteristics was not performed, and in the absence of such data it is very difficult to evaluate the effectiveness of this approach in increasing iron and zinc bioavailability without affecting plant performance.

On the other hand, a very novel and interesting approach has been used in maize and soybean to silence the genes involved in the biosynthesis of PA (Shi and others 2007). It was found that maize lpa1 mutants are defective in a MRP ATP-binding cassette (ABC) transporter that is more highly expressed in embryos, but also in immature endosperm, germinating seeds, and vegetative tissues. The expression of this transporter was silenced in an embryo-specific manner. The concentration of PA in seeds of maize transgenics was found to be reduced by up to 87% depending upon the transgenic line, and the transgenic plants were not affected in grain yield or seed germination in contrast to the lpa mutants. Similarly, silencing of MRP transporter in sorghum decreased the PA concentration in seeds by 80% to 86%, and a consequent increase in iron and zinc absorption was observed when analyzed in Caco-2 cell lines (Kruger and others 2013). Rice transgenics developed by silencing RIN01, a gene involved in the biosynthesis of PA, in embryo and aleurone layer-specific manner exhibited up to 67% decrease in PA and were unaffected in grain yield, seed germination, and plant performance (Kuwano and others 2009). These remarkable findings indicate the possibility to produce GMO cereal with low PA and without affecting agronomic performance by silencing the expression of transporters involved in the biosynthesis of PA. The difference in agronomic performance between the lpa mutants obtained through mutation breeding and low phytate transgenics is thought to be due to the tissue-specific silencing of the genes involved in biosynthesis of PA in case of transgenics.

What Should be the Compromise to Overcome Antinutritional but to still have Beneficial Properties of Phytic Acid?

For a long time PA was considered an undesirable plant metabolite for human nutrition, but a series of studies have challenged this concept. Almost all mammalian cells contain inositol phosphates, and PA comprises the bulk of the inositol phosphate content with intracellular concentration of about 100 μM (Sasakawa and others 1995), wherein it performs numerous cellular functions, such as protein trafficking and folding, cell division, cellular differentiation, export of mRNA from the nucleus of the cell, and DNA repair (Vucenik and Shamsuddin 2006). Recently, in different experimental models, PA was demonstrated as a broad-spectrum antineoplastic agent (colon cancer, breast cancer, prostate cancer, skin cancer) acting in different stages of cancer development and...
Increasing Enhancers of Iron and Zinc Absorption

Besides containing antinutrients, cereals do contain enhancers of iron and/or zinc absorption such as ascorbic acid and various prebiotics. Numerous studies have shown the dose-dependent positive effects of native or supplemented ascorbic acid for absorption of iron (Hurrel and Egli 2010). However, cooking, industrial processing, and storage degrade ascorbic acid and cancel out its mineral absorption effects (Teucher and others 2004). Prebiotics on the other hand are relatively resistant to such type of losses (Rakha and others 2010, 2011), hence are of special consideration in breeding programs aimed at enhancing bioavailability of iron and zinc, and thus will be discussed in detail in the following paragraphs.

Prebiotics are carbohydrates (nondigestible oligosaccharides [NDO] and lactulose) that selectively stimulate the growth and/or activity of a limited number of bacteria (probiotics) in the gastrointestinal tract and thereby exert beneficial effects on the host. A prebiotic is “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastro-intestinal microflora that confers benefits upon the host well-being and health” (Roberfroid 2007). With reference to mineral absorption, fructans are the most widely studied prebiotics. Fructans are polysaccharides made up of fructose units linked through $\beta-(2\rightarrow1)$ and/or $\beta-(2\rightarrow6)$ glycosidic bonds. Among fructans, inulins are the simplest and most widely studied. They are naturally present in various plant species such as chicory, onion, garlic, asparagus, leek, and so on (Venter 2007).

There exists notable evidence for the favorable influence of inulin on the absorption of minerals and trace elements, especially calcium and magnesium in living organisms (Scholz-Ahrens and others 2001; Bongers and van den Heuvel 2007; Greg Kelly 2009). An in vitro study aimed at investigating the influence of soluble dietary fibers on the availability of minerals revealed that inulin could significantly increase the availability of iron and zinc from dairy infant formulas (Bosscher and others 2003). In vivo studies have also shown promising results for the positive influence of inulin on iron and zinc absorption and are suggested to be dependent on experimental conditions such as dose of inulin, the duration of intake, subject animal, and the animal’s physiological status. Yap and others (2005) examined the dose-response effect of inulin (0.75, 1, and 1.25 g/d) on iron and zinc absorption in formula-fed infants. The authors found a significant increase in iron and zinc absorption in infants only at inulin doses of 1 and 0.75 g/d, respectively. Several studies conducted in rats have also shown that feeding rats with diet supplemented with inulin or other prebiotics could stimulate iron and/or zinc absorption (Afsana and others 2003; Asvarujanon and others 2005; Couday and others 2006; Patterson and others 2009; Har´a and others 2010). Pigs have also been used to explore the role of inulin in iron or zinc absorption because of very close similarity of their gastrointestinal tract anatomy and digestive physiology to that of human’s. In pigs, iron absorption was found to be enhanced when they were fed with a diet supplemented with inulin (Yasuda and others 2006; Patterson and others 2009). In one of the few studies performed with young healthy men to investigate the effect of different prebiotics including inulin, FOS, or galactooligosaccharides on iron absorption, no significant effect on iron absorption was observed (van den Heuvel and others 1998). However, studies involving different experimental conditions, iron and/or zinc status of the subjects, and ethnicity, age, and sex groups of humans are still missing to draw an inference about the influence of inulin on iron and/or zinc absorption.

The mechanisms through which inulin increases the bioavailability of intrinsic iron and zinc remain to be fully elucidated; microbial fermentation of these nondigestible carbohydrates in the large intestine is considered to play a significant role (Yeung and others 2005). Other explanations put forth include: improvement of gut health, alterations in mucus production, stimulation of gut-associated immune defence, health-promoting activity as antioxidant, and reactive oxygen species (ROS) scavengers in the gastrointestinal tract (Roberfroid 2005; Scholz-Ahrens and Schrezenmeir 2007; van den Ende and others 2011). The effect of dietary inulin on the gene expression of selected intestinal Fe transporters and binding proteins has also been examined, and it was found that the expression of DMT1, TFR, and ferritin in the colon of pigs was significantly induced by inulin (Tako and others 2008). Such a study yet remains to be carried out for zinc homeostasis-related genes to explore the influence of inulin on the expression of these genes in animals. These data may help us to better understand the mechanisms through which inulin enhances iron and zinc absorption.

Breeding Approaches to Increase Prebiotics

Until now, very few studies have been performed to explore the natural variation for the accumulation of fructans in cereal grains and thereby to dissect the genetic architecture of this trait. Wheat and rye are known to be better sources of fructans and contain 0.7% to 2.9% and 3.6% to 6.4% in grain (dry weight), respectively (Boskov Hansen and others 2003; Huynh and others 2008a).
Further analyses revealed that inulin concentration of wheat grain varies from 0.4 to 14.6 mg/g dry weight (Falcon 2011). Significant genetic variation in inulin concentration has also been reported in rice and maize, but their grain inulin concentration is far lower than those found in wheat and rye (Genec and others 2005). The highest rice variety contains approximately 7 times less inulin than the lowest wheat variety, and the highest maize variety contains 2 times less inulin than the lowest wheat variety. Genetic mapping studies were performed in wheat to identify the QTLs controlling the variation in grain fructan concentration (Huynh and others 2008b) and specifically inulin concentration (Falcon 2011). In total, 5 QTLs were found to be controlling the variation in grain fructan concentration in a doubled-haploid population of a cross between Berkut and Krichauff, two being the most important ones, located on chromosomes 6D and 7A, which explained 17% and 27%, respectively, of the total phenotypic variance (Huynh and others 2008b). A genetic mapping study performed by Falcon (2011) using a doubled-haploid population generated from a cross between AC Reed and Grandin showed that the 2 QTLs controlling grain inulin concentration in wheat are located on chromosomes 2B and 5B and explained approximately 20% and 15%, respectively, of the total phenotypic variance. The presence of approximately 4-fold variation in the grain inulin concentration and the modest effect of QTLs controlling this variation indicate that inulin concentration can be significantly ameliorated in wheat grains through molecular breeding. Yet, numerous studies using different genetic backgrounds of wheat need to be performed under variable environmental conditions to identify robust QTLs that can be of interest to a wider scientific community. In the case of rice, an ideal starting genetic material could be a large set of rice accessions designed by Zhao and others (2011) for which genotypic data are already available. These accessions can be phenotyped under variable environmental conditions to identify genomic regions controlling variation of grain inulin concentration through association mapping. The availability of Nested Assn. Mapping population in the case of maize (McMullen and others 2009) is considered highly valuable material to identify genetic determinants of inulin accumulation in maize grains. The way forward would be to identify the markers that can help molecular breeding programs aiming at increasing grain inulin concentration.

Increasing Grain Iron and Zinc Concentration

In order to increase the accumulation of iron and zinc in cereal edible parts, we need to have good understanding of physiological, genetic, and molecular mechanisms involved in iron and zinc homeostasis in plants. Numerous reviews have been published regarding iron and/or zinc homeostasis in plants (Grotz and Guerinot 2006; Briat and others 2007; Palmgren and others 2008; Jeong and Guerinot 2009; Palmer and Guerinot 2009; Lee and others 2012; Murgia and others 2012; Sinclair and Krämer 2012; Sperrotto and others 2012). Here, instead of discussing in detail the iron and zinc homeostasis networks in plants, attention will be focused on the strategies that can be chosen to develop cereal crops with a high accumulation of iron and zinc in their edible parts. Moreover, as there exists crosstalk between different minerals in crops which could be synergistic or antagonistic depending upon the minerals under consideration, we thus advocate looking into the opportunities and challenges related to accumulation of iron and zinc, and also other heavy metals such as cadmium and arsenic at the same time. The accumulation of arsenic is of particular concern in rice because this crop is mainly cultivated under anaerobic conditions where arsenite (As(III)) is more available (Takahashi and others 2004).

Breeding Approaches to Increase Iron and Zinc Accumulation

Natural variation for accumulation of iron and zinc in grains of cereals has been explored to a great extent, and significant differences for iron and zinc concentration in grains of cereals have been observed. The grain iron, as well as zinc concentration, varies 2-fold in cultivated wheat species (Triticum aestivum, Triticum turgidum spp. durum) as well as in their wild relatives (Xu and others 2011). Compared to cultivated wheat, wild relatives belonging to the genus Aegilops can accumulate significantly higher iron and zinc in their grains (Chhuneja and others 2006). Thus, it could be hypothesized that synthetic hexaploid wheat, developed by crossing tetraploid wheat cultivars with diploid wild relatives, would contain higher iron and zinc in the grains. Synthetic hexaploids developed by crossing Triticum turgidum spp. durum with Ae. tauschii indeed accumulated about 30% higher iron and zinc in the grains (Calderini and Ortiz-Monasterio 2003). Unfortunately, grain yield of these synthetics was reduced by about 25%, which is by no means desirable for farmers. Such negative correlations between iron and zinc concentration and grain yield have been reported in numerous studies, although the strength was greatly influenced by the environment (Oury and others 2006; Morgounov and others 2007; Ficco and others 2009; Zhao and others 2009). Several QTLs controlling iron and zinc concentration in wheat grains have been mapped using RIL or DH population issued from crosses of different parents (Dustefeld and others 2007; Shi and others 2008; Peleg and others 2009; Tiwari and others 2009).

Genetic Engineering Approaches to Increase Prebiotics

As mentioned earlier, rice and maize varieties analyzed to date contain lower grain fructan, but it is believed that exploring new materials like landraces, wild relatives, and so on may help identify the sources that can be used in breeding programs to increase grain fructan content. As for now, rice and maize transgenics have been developed that can accumulate high amounts of fructan in grains. Fructans are produced by the combined action of various fructosyltransferases (FTs) (Pan and others 2009). The enzyme 1-SST catalyzes the initial fructosyl transfer between 2 sucrose molecules. Then FTs (1–FFT, 6G–FFT, 6–SFT, and so on) catalyze chain elongation adding β-(2→1)- or β-(2→6)-linked fructose units. Inulin-type fructan biosynthesis in plants is generally believed to occur through the collective action of 2 vacuolar enzymes, 1-SST and 1–FFT. The expression of these enzymes, 1-SST and 1–FFT, from Jerusalem artichoke (a high-inulin-accumulating plant) in high-sucrose maize under the control of an endosperm-specific promoter increased the fructan content by 2-fold (Stoop and others 2007). Moreover, kernel development and seed germination of these transgenic maize plants were not hampered. The overexpression of 1-SST enzyme from Jerusalem artichoke as well as Yacon (another high-inulin-accumulating plant) in rice under the control of a constitutive promoter significantly enhanced the production of fructan in plant tissues of transgenic rice (Pan and others 2009). Constitutively, overexpression of 1-SST enzyme of wheat in rice could increase seed fructan of rice transgenics, with a slight decrease in seed weight (Kawakami and others 2008).

It is likely that increasing fructan content could have a positive effect on iron and zinc absorption. Beside the nutritional aspects, agronomic performance of the plants should also be considered while developing high-fructan-accumulating cereals.
Remarkably, 3 of these QTL mapping studies, performed over different years and under various agro-ecological conditions, revealed a common QTL on chromosome 7A around 70 centi-morgan to be contributing about 10% of the phenotypic variance to the grain iron and zinc concentration. Further, positional cloning of Gpc-B1, a wheat quantitative trait locus associated with increased iron and zinc concentration, identified a no-apical meristem (NAM) transcription factor to be responsible for this variation (Uauy and others 2006). QTL mapping studies have also been performed in rice using RIL populations (Lu and others 2008; Garcia-Oliveira and others 2009; Norton and others 2010; Anuradha and others 2012). QTLs affecting both iron and zinc concentration in grains are more often co-localized on chromosome 7 and chromosome 12 in rice. The genes underlying these QTLs have not yet been identified. Co-localization of QTLs affecting grain iron and zinc concentration has also been found in maize mapping populations (Qin and others 2012). These are very encouraging findings because it suggests that iron and zinc concentration in cereal grains can be increased simultaneously by exploiting the same chromosomal regions in MAS. However, QTL mapping studies performed until now have only addressed variation for accumulation of iron and zinc in whole-grains, and none of the QTL mapping studies has been undertaken to identify the QTLs controlling variation for the accumulation of these minerals in the endosperm of cereals. It is important to note here that more than 75% of minerals accumulated in whole grain are lost during milling (Slavin and others 2000; Barry 2006; Ozturk and others 2006). Moreover, the accumulation of these minerals in grains of cereals is significantly influenced by the environment (Ouiry and others 2006; Morgounov and others 2007; Lu and others 2008; Filco and others 2009; Garcia-Oliveira and others 2009; Zhao and others 2009; Norton and others 2010; Langhao and others 2011; Anuradha and others 2012; Qin and others 2012; Simić and others 2012). Thus, efforts are required to map QTLs controlling iron and zinc accumulation in endosperm of cereal grains, and to identify genetic determinants and developing markers that would be of use in various genetic backgrounds and under variable environmental conditions. An important mechanism that scientists may focus to exploit is transgression, because positive transgressive segregants beyond what is found in the parents. In addition, characterizing cereal germplasms, including landraces and wild relatives, can also help finding genotypes with higher iron and zinc concentration that can be used in breeding programs.

**Genetic Engineering Approaches to Increase Iron and Zinc Accumulation**

The approach heavily relies on the available knowledge of iron and zinc homeostasis mechanisms in plants in general and cereals in particular. As far as cereals are concerned there are 3 major mechanisms, modulation of which can help to increase the accumulation of iron and/or zinc in edible portions of grains; the 1st being uptake and remobilization in roots, the 2nd being xylem-loading, and the 3rd one is remobilization of these minerals into the edible parts. Generally, higher plants rely on reduction and/or chelation-based strategies to take up iron from the soil. The description of these strategies is beyond the scope of this review and can be found elsewhere (Bauer and Heli 2006). Briefly, reduction-based strategy is characterized by the reduction of Fe (III) to take up Fe (II) accompanied by soil acidification and other physiological and morphological reactions. This strategy is prevalent in dicotyledonous and non-graminaceous plants. However, rice is an exception to this rule where reduction-based strategy to take up iron has also been reported that is considered advantageous for acquisition of iron under submerged field conditions (Ishimaru and others 2006; Cheng and others 2007). Cereal crops typically use a chelation-based strategy to take up iron and zinc from the soil (Römheld and Marschner 1986), which employs the release of phytosiderophores (PS) into the rhizosphere to mobilize iron and zinc thereby enhancing their uptake. PS are hexadentate metal chelators with high affinity to form complexes with Fe (III). These Fe (III)–PS complexes are thereby taken up by plants, thanks to the members of YSL/YSL (Yellow Stripe Like) gene family (Curie and others 2001; Inou and others 2009). Moreover, the ability of PS to chelate zinc has also been reported (von Wirén and others 2000). The molecular mechanisms underlying the release of PS in rice were unraveled recently, and it was shown that the expression of TOM1 gene is associated with the efflux of deoxymugenic acid (DMA), a primary member of the PS family (Nozoye and others 2011). The release of DMA has been reported to be enhanced under iron and zinc deficiency conditions in diploid, tetraploid, and hexaploid wheats (Today and others 2001). Significant correlation observed between the DMA release and iron and zinc deficiency tolerance of different wheat genotypes suggested an important role of DMA in iron and zinc uptake in wheat. In rice, quite consistent results have been obtained with respect to the positive role of DMA in iron acquisition (Suzuki and others 2008), however, contradictory results have been obtained with zinc. However, modeling performed to illustrate the role of DMA in zinc uptake indicated that measured rates of DMA secretion reported for rice are sufficient to significantly increase zinc uptake and to explain the differences between the genotypes (Arnold and others 2010). However, a previous physiological study performed in rice attributed the role of zinc translocation to DMA rather than zinc uptake based on the fact that the rice plants grown under zinc-deficient conditions when supplied with free zinc or zinc-DMA complex preferred to take up free zinc (Suzuki and others 2008).

In addition to these PS, zinc transport in roots is also thought to be mediated by other intracellular high-affinity binding sites like phytochelatins (PCs) and metallothioneins (MTs) (Paling and others 2008). These metal chelators have the capacity to bind a variety of heavy metals, including iron and zinc, thus keeping the free-metal concentration to be very low, and interact with and donate metals to apometalloproteins or transport proteins that mediate the sequestration or efflux of metal ions (Callahan and others 2006). PCs are oligomers of glutathione, produced by the enzyme phytochelatin synthase. Recently, a role of PCs in zinc sequestration has been described in different mutants of yeast and A. thaliana (Tennstedt and others 2009). MTs are small cysteine-rich proteins involved in metal homeostasis. In vitro assays revealed that AtMT2 could chelate zinc (Robinson and others 1996). Functional heterologous expression of AtMT2 partly complemented zinc hypersensitivity in mutants of Synephocclus. A recent study in rice also reported the involvement of OsMT1a in zinc homeostasis (Yang and others 2009). OsMT1a was found to be predominantly expressed in the roots and was induced by zinc treatment.
Overexpression of OsMT1a resulted in approximately 1.5- and 2.5-fold increases in zinc accumulation in the OsMT1a overexpressing rice lines and yeast, respectively.

Proper functioning of cellular machinery requires a certain amount of zinc in the cytosol to serve the needs of cell organelles. So, the zinc in excess of the nutritional needs might be transported to the vacuole to avoid cytotoxic effects which can be remobilized when required. Vacuoles are assumed to be major sites of zinc sequestration and thereby detoxification (Martinoia and others 2007). In A. thaliana, Metal Tolerance Protein 1 (MTP1) and Metal Tolerance Protein 3 (MTP3), members of the Cation Diffusion Facilitator (CDF) family have been found to be implicated in vacuolar zinc sequestration (Desbrosses-Fonrouge and others 2005; Arrivault and others 2006). Ectopic overexpression of AtMTP1 enhanced zinc tolerance and increased zinc accumulation of A. thaliana transgenics (van der Zaal and others 1999). Functional heterologous expressions and transcript accumulation analyses in response to zinc do provide evidence for the role of MTP1 in zinc tolerance in zinc hyperaccumulators (Persans and others 2001; Dräger and others 2004; Shahzad and others 2010, 2012). A. thaliana transgenics overexpressing AtMTP3 under the control of 35S promoter displayed enhanced zinc tolerance and increased zinc accumulation (Arrivault and others 2006). Recently, A. thaliana Heavy Metal Associated 3 (AtHMA3) protein belonging to the P(1b-2) subgroup of the P-type ATPase family was also shown to be involved in vacuolar zinc sequestration (Morel and others 2009). A. thaliana lines overexpressing AtHMA3 were more tolerant to zinc as compared to the wild type. Conversely, a T-DNA AtHMA3 mutant was sensitive to zinc. The accurate expression of Zinc-Induced Facilitator1 (ZIF1), a vacuolar membrane major facilitator superfamily protein required for basal Zn tolerance, was also shown to be very crucial for both iron and zinc homeostasis (Haydon and others 2012). All these findings support the critical role of vacuoles that needs to be considered while developing iron and zinc biofortified cereals. Under zinc-deficient conditions the zinc stored in the vacuole has to be remobilized to the cytosol to serve the needs of the cell. We still have very poor knowledge of the transporters that could be involved in the release of zinc from the vacuole under zinc-limiting conditions in cereals. It is thought that possible candidates should belong to either ZIP family transporters, YSL family, or Natural Resistance-Associated Macrophage Protein (NRAMP). A transporter involved in iron remobilization, AtNRAMP4, might also be involved in zinc remobilization. AtNRAMP4 is a tonoplast member of the NRAMP gene family (Langur and others 2005). Heterologous expression of AtNRAMP4 complemented the growth phenotype of zrt1 zrt2 S. cerevisiae mutant suggesting its role in zinc transport across membranes (Langur and others 2004). Transcript analysis revealed that AtNRAMP4 was induced in the roots under excess zinc conditions (van de Mortel and Aarts 2006). Localization of AtNRAMP4 promoter activity was determined using AtNRAMP4 promoter::GUS constructs (Langur and others 2005). It was shown that GUS activity was detected in roots as well as in shoots under iron deficient conditions, and in roots staining was stronger than in the phloem of stele. The previous findings have established the role of AtNRAMP4 in the remobilization of iron from the vacuole. Whether NRAMP4 or some other member of the NRAMP family could also play a role in zinc remobilization still remains to be carefully examined.

Despite the availability of knowledge about the involvement of different genes in iron and/or zinc uptake and remobilization in roots, the scientific community has largely focused attention on exploiting NAs (precursors of mugenic acid-family phytosiderophores) for developing cereal transgenics able to accumulate high iron and zinc in edible portions of grains. Expression of either endogenous or exogenous NAs (precursors of mugenic acid-family phytosiderophores) under the control of tissue-specific or constitutive promoters have interestingly proved to be very effective to increase iron and/or zinc accumulation in polished rice grains, at least under controlled growth conditions. Transgenic rice lines expressing nicotianamine synthase (NAS) gene of Hordeum vulgare accumulated 2- to 3-fold higher iron and zinc in polished rice grains (Masuda and others 2009). Endosperm-specific overexpression of endogenous rice NAS (OsNAS1) resulted in an even higher increase (5-fold) in iron accumulation in polished rice grains (Zheng and others 2010). Comparative analysis of 3 rice NAS homologous proteins, OsNAS1, OsNAS2, and OsNAS3, showed OsNAS2 to be the most effective in increasing iron as well as zinc concentration in polished rice grains (Johnson and others 2011). Although more than a 2-fold increase in iron and zinc concentration in polished rice grains was consistently found by overexpressing NAS genes, but when grown under field conditions only about 1.5-fold increase was observed in polished rice grains of these transgenics (Masuda and others 2012). Further, an innovative strategy to develop a rice transgenic with high accumulation of iron in polished rice grains was recently developed (Masuda and others 2013). The authors introduced the soybean ferritin gene (SoyferH2) driven by endosperm-specific promoters, along with the barley nicotianamine synthase gene (HvNAS1), two nicotianamine aminotransferase genes (HvNAAT-A and -B), and a mugineic acid synthase gene (IDS3) to enhance mugineic acid production in rice plants while using a marker-free vector as a means of increasing public acceptance. Interestingly, the iron accumulation in polished grains of this pyramided transgenic was increased by 2.5- to 4-fold depending upon the soil type.

Iron and zinc xylem loading and transport

Because iron is poorly soluble and highly reactive, its transport within the plant body must be associated with suitable chelating molecules and proper control of redox states between ferrous and ferric forms (Kobayashi and Nishizawa 2012). The mechanism controlling long-distance iron transport is barely known. Citrate was shown to be a principal chelating and trafficking agent of iron in xylem sap of tomato (Rellán-Alvarez and others 2010). Citrate efflux into the xylem is controlled by Ferric Reductase Defective 3 (FRD3), an Arabidopsis transporter of the Multidrug and Toxin Efflux (MATE) family (Durrett and others 2007). A. thaliana frd3 knockout mutant exhibited severe alterations in iron localization, and shoot protoplasts of this mutant contained one-half of the iron as compared to the wild type (Green and Rogers 2004), confirming the role of FRD3 in xylem-loading of iron. Interestingly, a forward genetics approach performed to identify the gene controlling zinc tolerance in Arabidopsis revealed that FRD3 in addition to iron is involved in loading zinc into the xylem (Pineau and others 2012), suggesting that there exist cross-homeostasis between the 2 minerals for xylem-loading. Knocking-out of a homologue of FRD3 in rice also resulted in a decrease in leaf iron and an increase in leaf zinc concentration (Yokosho and others 2009). Besides, specific molecular mechanisms for zinc loading into xylem have also been reported. The plasma membrane transporters, AthMA2 and AthMA4, members of PIB-ATPases, have been well documented for their role in zinc loading into the xylem (Hussain and others 2004; Verret and others 2004). Zinc accumulation was increased 2-fold in...
roots and decreased by 2-fold in shoots of the *A. thaliana hma2 hma4* double mutants (Hussain and others 2004). Overexpression of AtHMA4 in *A. thaliana* resulted in a 2-fold increase in zinc contents in leaves, whereas no significant change was observed in root zinc contents (Verret and others 2004). HMA4 was shown to be highly overexpressed in *A. halleri* as compared to *A. thaliana* (Hanikenne and others 2008). In *A. halleri*, knock-down of HMA4 expression by 45% to 10% resulted in a decrease of 12% to 35% shoot zinc concentration and an increase of 49% to 134% root zinc concentration as compared to wild-type plants. In the roots of *A. thaliana* and *A. halleri*-specific miRNA, activity of HMA4 was observed in root pericycle and xylem parenchyma (Hanikenne and others 2008). Taken together, these data strongly support the role of HMA2 and HMA4 in zinc loading into the xylem. However, these transporters are not specific for zinc-loading, and are also involved in cadmium transport as shown in *A. thaliana* and *A. halleri* (Verret and others 2004; Hanikenne and others 2008).

Unlike dicot model plants, few reports on HMA4s are available in cereals. The orthologue of HMA2 in rice (*OhsHMA2*) has been shown to be involved in zinc and cadmium loading into the xylem (Takahashi and others 2012). The nonspecificity of HMA2/HMA4 for zinc and cadmium transport seems a serious shortcoming for using HMA2/HMA4 in biofortification programs. Yet, it might be possible to identify the domains in the protein that are specific to zinc transport and to engineer the protein to transport only zinc, and use the engineered protein for biofortification of cereals (Rouached 2012). Another plasma membrane transporter belonging to the ZIP family AtIRT3 is also thought to facilitate zinc-loading into the xylem under zinc-limiting conditions (Lin and others 2009). AtIRT3 is induced under zinc-deficient conditions and repressed by excessive zinc. AtIRT3 promoter was ubiquitously active, and activity was especially observed in root stele and passage cells. *IRT3* from *A. thaliana* functionally complemented the zinc uptake of *zrt1 zrt2 S. cerevisiae* mutant. An increase in zinc accumulation was detected in the shoots of *AtIRT3* overexpressing *A. thaliana* lines. The expression of *IRT3* was detected to be higher in the zinc-hypertolerant and hyperaccumulator *A. halleri* than in the zinc-sensitive and nonaccumulator *A. thaliana*. These findings indicate a positive association between *IRT3* expression and zinc accumulation. Once loaded into the dead xylem, free zinc may move upward to shoots along the xylem sap. However, nicotianamine ligands of zinc have also been proposed to be involved in the long-distance transport of zinc in dead xylem (Suzuki and others 2008). These mechanisms provide an interesting opportunity to develop iron and zinc biofortified cereals using a genetic engineering approach, but have not been explored to date.

**Iron and zinc remobilization to seeds**

To reach the final destination iron and zinc have to be unloaded from the dead xylem. The xylem parenchyma has to actively take these minerals up to desired levels in the edible parts of cereals by these strategies under field conditions. Moreover, it is not clearly identified how agronomic or genetic biofortification interventions affect the accumulation of toxic heavy metals such as cadmium and arsenic in edible portions of cereal grains. Future research should involve analyzing the accumulation of iron and zinc, as well as other heavy metals, in the edible parts of cereal grains rather than whole grains. It is also advised that the impact of genetic modifications on the agronomic performance of crops, including grain yield, drought tolerance, insect resistance, disease resistance, and so on should also be assessed. In addition, the focus should be on studies involving field crop trials and human beings as experimental subjects to analyze the effectiveness of agronomic or genetic biofortification.
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