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Nutritionally Enhanced Staple Food Crops

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ABSTRACT

Crop biofortification is a sustainable and cost-effective strategy to address malnutrition in developing countries. This review synthesizes the progress toward developing seed micronutrient-dense cereals and legumes cultivars by exploiting natural genetic variation using conventional breeding and/or transgenic technology, and discusses the associated issues to strengthen crop biofortification research and development. Some major QTL for seed iron and zinc, seed phosphorus, and seed phytate in common bean, rice, and wheat have been mapped. An iron reductase QTL associated with seed-iron QTL is found in

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common bean where the genes coding for candidate enzymes involved in phytic acid synthesis have also been mapped. Candidate genes for *lpa* cosegregate with mutant phenotypes identified in rice and soybean. The *Gpc-B1* locus in wild emmer wheat accelerates senescence and increases nutrient remobilization from leaves to developing seeds, and another gene named *TtNAM-B1* affecting these traits has been cloned. Seed iron-dense common bean and rice in Latin America; seed iron-dense common bean in eastern and southern Africa; seed iron-dense rice in the Philippines; and β-carotene-rich sweet potato in Latin America have been released for cultivation, with more nutritionally enhanced lines in pipeline for release, mostly in developing countries. Exceptionally large variations in β-carotene have been reported in temperate maize germplasm, which have been transferred into tropical maize hybrids, being evaluated prior to their release in Mexico and in some countries in Africa. The high β-carotene trait in 'Golden Rice 2' is being introgressed into several Asian rice cultivars. At CIMMYT, molecular markers for *LycE* and *HydB* are fully implemented, have accelerated breeding by one season, and substantially enhance efficiency and effectiveness of high-provitamin A maize breeding. Marker-assisted selection has been successfully employed to transfer low phytate into improved soybean cultivars. Biofortified crops are being investigated for efficacy with human and animal systems. Transgenic rice containing *AtNAS* and *Pvferritin* have increased seed iron several folds, while transgenic maize containing *phyA2* shows high phytase activity and reduced phytic acid compared to wild types. Maize transgenics containing five carotenoid genes show higher accumulation of β-carotene with no adverse effects on agronomic traits. A rigorous assessment is suggested to identify advantages of biofortified food for human health. Several issues, in addition to the above, related to biofortification, have been highlighted for furthering biofortification research in staple food crops. Biofortification has been included as core breeding activity in some countries in Latin America to ensure that newly developed crop cultivars meet nutritional needs of humans.

KEYWORDS: applied genomics; bioavailability; biosafety; β-carotene; crop biofortification; micronutrients

ABBREVIATIONS

AAS	Atomic absorption spectrophotometer
AGP	α_1-Acid glyco protein
β-carotene	Beta carotene
BMI	Body mass index
CB	Conventional bean
cDNAs	Complementary deoxyribonucleic acids
CGIAR	Consultative Group on International Agricultural Research
CIAT	Centro Internacional de Agricultura Tropical
CIMMYT	International Maize and Wheat Improvement Center
CM	Conventional maize
CRP	C-reactive protein
crtRB1	β-Carotene hydroxylase 1
DALYs	Disability-adjusted life years
DMA	2′-Deoxymugineic acid
DNA	Deoxyribonucleic acid
E	Environment
EDX	Energy dispersive X-ray microanalysis
epi-HDMA	3-Epihydroxy-2′-deoxymugineic acid
epi-HMA	3-Epihydroxymugineic acid
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FISH	Fluorescent *in situ* hybridization
FIGS	Focused Identification of Germplasm Selection
G	Genotype
GEI	Genotype environment interaction
GI	Gastrointestinal
GISH	Genomic *in situ* hybridization
GM	Genetically modified
Gpx1	Glutathione peroxidase-1
Hb	Hemoglobin
HI	Harvest index
HPLC	High performance liquid chromatography
ICAR	Indian Council of Agricultural Research
ICARDA	International Center for Agricultural Research in the Dry Areas
ICP	Inductively coupled plasma
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IITA	International Institute of Tropical Agriculture

IRA	Iron reductase activity
IRRI	International Rice Research Institute
LG	Linkage group
LCYE	Lycopene ε-cyclase
LMWOA	Low molecular weight organic acids
lp	Low phytate
MA	Mugineic acid
MABC	Marker-assisted backcrossing
MAS	Marker-assisted selection
MGT	Multigene transfer
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
nano-SIMS	Nano-secondary ion mass spectrometry
NARs	National agricultural research systems
NEB	Nutritionally enhanced bean
NEM	Nutritionally enhanced maize
NIRS	Near infrared reflectance spectroscopy
OFSP	Orange-fleshed sweet potato
PFDS	Public food distribution system
Pi	Inorganic phosphorus
PIXE	Proton-induced X-ray emission
PS	Phytosiderophore
QPM	Quality protein maize
QTL	Quantitative trait loci
STEM	Scanning and transmission electron microscopy
VPKAS	Vivekananda Parvatiya Krishi Anusandhan Sansthan
WT	Wild type
μ-XRF	Micro X-ray fluorescence

I. INTRODUCTION

The world's population is projected to grow by 34% from the current 6.8 billion to 9.1 billion in 2050, with nearly all of this increase in developing countries. Meanwhile global demand for food during the same period is projected to be 70% higher than today, involving an additional annual consumption of nearly 1 billion tonnes of cereals for food and feed and 200 million tonnes of meat (World Summit on Food Security 2009, here cited as WSFS 2009). The accumulated evidence reveals that not only crop yields decline at temperatures much above 30°C (Schlenker and Roberts 2009) but also the produce from such crops is less nutritious (Qaderi and Reid 2009). Climate models predict that

warmer temperatures and increases in the frequency and duration of drought during the 21st century will have net negative effects on agricultural productivity. It is predicted that CO_2 level in the atmosphere is expected to rise due to climate change and studies in wheat, barley, rice, and rapeseed have shown significant declines in total protein and essential amino acids, as well as a range of macro- and micronutrients (Högy and Fangmeier 2008; Taub et al. 2008; Högy et al. 2009, 2010; Erbs et al. 2010). Furthermore, the negative impacts of climate change on soil fertility and mineral nutrition of crops will far exceed beneficial effects, with serious consequences to food security in developing countries (St. Clair and Lynch 2010). To feed ~9 billion people by the middle of 21st century, the production of high-quality food must increase with reduced inputs. Plant breeders need to focus on traits with the greatest potential including resistance/tolerance to biotic and abiotic stresses to increase yield. New technologies can help accelerate breeding in such a way that it increases the available genetic diversity to improve food and nutritional security (Fedoroff et al. 2010; Tester and Langridge 2010).

Micronutrient malnutrition arising from Zn and Fe deficiencies alone affect over 3 billion people around the world (http://www.unscn.org). Nearly 500,000 children <5 years of age die annually because of Zn and Fe deficiencies (Black et al. 2008). Furthermore, micronutrient malnutrition is accompanied by serious physical incapacity, mental impairment, decreased health, and parasitic diseases. The number of chronically undernourished people has risen from 842 million at the beginning of the 1990s to over 1 billion in 2009 (World Summit on Food Security 2009; Barrett 2010). Fighting malnutrition is an integral component of three of the eight millennium development goals, that is, eradication of extreme poverty and hunger, reduction of child mortality, and improvement of maternal health (http://www.UNESCAP.ORG/publication/survey2002/suro2-ii.pdf), with World Summit on Food Security endorsing to halt immediately the increase in and to significantly reduce the number of people suffering from hunger, malnutrition, and food insecurity, and reinforce efforts to meet by 2015 the targets of the millennium development goals (Sachs and McArthur 2005).

Widespread micronutrient malnutrition results in an enormous negative socio-economic impact at the individual, community, and national levels (Darnton-Hill et al. 2005; Stein 2010). Among the 26 major risk factors of the global burden of disease estimates, iron deficiency ranks 9th, zinc deficiency 11th, and vitamin A deficiency 13th (Ezzati et al. 2002). Disability-adjusted life years (DALYs) are

used to establish the burden of a disease by measuring the health loss through mortality and morbidity in a single index (Murray and Lopez 1996). In India, over 9 million DALYs are lost annually due to iron, zinc, and vitamin A deficiencies, with iron deficiency alone contributing to 4 million DALYs lost (Qaim et al. 2007). The disease burden associated with iron deficiency in India could be reduced by 19%–58% by crop biofortification (Stein et al. 2008). Likewise, Stein et al. (2007) reports annually 2.8 million DALYs lost due to zinc deficiency in India. Zinc biofortified rice and wheat may reduce this burden by 20%–51% and save 0.6–1.4 million DALYs each year. The costs for saving one DALY through crop biofortification amount to US$ 0.73–7.31, which is very cost-effective and lower than that of most other micronutrient interventions (Stein 2006; Ma et al. 2007). More recently, Meenakshi et al. (2010) concluded that overall, biofortification can make a significant impact on reducing the burden of micronutrient deficiencies in the developing world in a highly cost-effective manner; however, the impacts differ depending on the combination of crop, micronutrient and country, and the major reasons underlying these differences are identified to inform policy.

In the past, food fortification, diet diversification, and nutrient supplementation have been most frequently used as public health interventions to reduce micronutrient-induced morbidity and mortality worldwide (Suharno et al. 1993; Haider et al. 2003; Brown et al. 2007; Wienecke and Gruenwald 2007; Casey et al. 2010; Eneroth et al. 2010). However, these approaches have had only limited success and could not by themselves attain sufficiently millennium development goals mainly because such interventions require infrastructure, continuous flow of resources, purchasing power, or access to markets and health care systems to their success, often not available to people living in remote areas (Underwood 1999; Imhoff-Kunsch et al. 2007; Ssemakula and Pfeiffer 2011).

The rural-based diets are predominantly composed of cereals and legumes, with limited access to fruits and vegetables that are regarded as rich sources of minerals and vitamins. The consumption of fruits and vegetables along with staple foods facilitates more bioaccessible micronutrients that otherwise would be inaccessible due to the presence of antinutrients, such as phytates and tannins (Ballot et al. 1987; Ali and Tsou 1997; Jansen van Rensburg et al. 2004; Flyman and Afolayan 2006; Baruah and Borah 2009). Integrating micronutrient-rich foods such as legumes, vegetables, and fruits into diets is the most practical and sustainable way to alleviate micronutrient deficiency.

An alternative (or complement) to the above approaches is to use plant breeding to naturally fortify commonly consumed staple crops with micronutrients, a process known as genetic biofortification (Bouis 2003). Biofortification has the potential to help alleviate the suffering, death, disability, and failure to achieve full human potential that result from micronutrient deficiency-related diseases (Haas et al. 2005; Campos-Bowers and Wittenmyer 2007; Rosado et al. 2009; Arsenault et al. 2010; Bouis and Islam 2011). In summary, therefore, biofortification refers to the development of micronutrient-dense staple crops using traditional breeding practices or biotechnology (Bouis 2003). Crop biofortification offers the unique opportunity to create international public goods (IPGs) by exploiting natural genetic variation to develop seed mineral-dense crops. A 10-year Consultative Group on International Agricultural Research (CGIAR) Challenge Program on "Biofortified Crops for Improved Human Nutrition" was initiated in 2004 with support from the Bill and Melinda Gates Foundation, the World Bank, and USAID to improve the health of poor people by breeding staple food crops that are rich in iron, zinc, and vitamin A (β-carotene), with full-time breeding programs initiated on rice, wheat, maize, cassava, sweet potato, and common beans consumed by the many of the world's poor (www.harvestplus.org). Prebreeding feasibility studies have been performed on banana, barley, cowpea, groundnut (peanut), lentil, pearl millet, pigeon pea, plantains, potatoes, sorghum, and yams. This program is a multinational, multisectoral, multidisciplinary, multicrop, multinutrient, and multipartnered undertaking (Pfeiffer and McClafferty 2007; Bouis and Islam 2011).

In agronomic terms, the mineral-dense seeds of biofortified cultivars often produce more viable and vigorous seedlings and such plants are more efficient in mineral uptake thus improving disease resistance and some growth characteristics (Rengel and Graham 1995; Graham and Welch 1996). Welch and Graham (2005) report that the improved seedling vigor is associated with the production of more and longer roots under micronutrient-deficient conditions, allowing seedlings to scavenge more soil volume for micronutrients and water early in growth, an advantage that can lead to improved yields compared to seeds with low micronutrient stores grown on the same soils. Additionally, greater stress tolerance has been noticed in seedlings grown from micronutrient-dense seeds resulting in higher agricultural production (Welch 1986; Cakmak 2008). Agronomic biofortification is not the subject of this review but includes cropping systems diversification, fertilizer enrichment, seed priming, or use of soil microorganisms such as mycorrhizal fungi to improve soil rhizosphere environments for

better acquisition of immobile mineral elements from the root zones (He and Nara 2007; Cakmak 2008; Cavagnaro 2008; Khoshgoftarmanesh et al. 2010).

A final issue of utmost importance is adoption of biofortified cultivars. The farmer's perception of adoptability of a new cultivar must be taken into account and is such that new cultivar should show yield superiority and/or tolerance to stresses and produce more than the existing cultivars with respect to seed yield and other characteristics such as taste, size, color, and flavor. For biofortified crops, there should be assured market and the produce must earn farmers more income if the cultivar is to be widely adopted and therefore the nutritional benefit is to be widespread. Public investments in research to create biofortified crops have increased in recent times because of the growing awareness about the importance of micronutrient deficiencies and associated adverse health effects. Consumer acceptance of the biofortified crops could be an issue if the new intervention changes the appearance or taste of the food crops (Wolson 2007); however, this may not be the case for the crops biofortified with so-called "invisible traits" such as iron or zinc. Crops fortified with β-carotene exhibit a deep yellow to orange color as seen in the case of golden rice, orange-fleshed sweet potato (OFSP); and yellow cassava (Pray et al. 2007; Ramaswami 2007), which may limit their acceptance as food. The biosafety issues of nutritionally enhanced transgenic crops, whether this is for iron and zinc or for provitamin A, is another issue that should be adequately addressed to allay consumers concern about the safety of the genetically modified (GM) crops.

This review focuses on the progress realized toward developing seed nutrient-dense (Fe, Zn, and β-carotene) cultivars of cereals and legumes by exploiting natural genetic variation using conventional breeding and/or transgenic technology. Associated with this we also discuss other critical issues such as: (1) the need to develop rapid, cost-effective, and accurate phenotypic screening tools/techniques; (2) traits associated with increased nutrient use efficiency, storage, and reallocation of nutrients; (3) models and biomarkers for assessing bioavailability and diagnosing micronutrient deficiency; (4) factors associated with bioavailability of micronutrients; (5) micronutrient distribution in seeds; (6) genotype (G) by environment interaction and the nature of associations (and trade-offs) between micronutrients and agronomic traits; (7) markers and quantitative trait loci (QTL) associated with increased micronutrients; and (8) biosafety issues associated with the use of genetically modified crops for biofortification.

II. BIOMARKERS FOR ASSESSING NUTRITIONAL STATUS

Biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic and pathogenic processes, or pharmacologic responses to a therapeutic intervention (http://www.everythingbio.com/glos/definition.phf?ID=3716). The use of a biomarker that reflects the changes in the micronutrient status can indeed facilitate the understanding of the relationships between dietary micronutrient intake and the status, and those between micronutrient status and health. Anthropometry (measuring weights and heights and other body measurements) and biochemical tests (mostly blood, sometimes urine) are the two most often used methods to assess the nutritional status of the populations or individuals. Anthropometry is the universally applicable, inexpensive, and noninvasive technique for the assessment of growth and nutrition. The basic anthropometric measurements include the body mass index ($BMI = weight/height^{-2}$) and the waist circumference (either alone or expressed against hip circumference).

The BMI is the most useful parameter as a direct measure of over- or underweight as classified by the World Health Organization (WHO): BMI < 18.50 indicates underweight, 18.5–24.99 normal range, 25.00–29.99 overweight, >30.00 obese, and >40.00 very severely obese. The waist circumference correlates quite closely with the BMI. The weight-for-age is the primary index, based on the U.S. Center for Disease Control growth reference, for measuring children's growth. In the weight-for-age graphs, the average is the 50th percentile (i.e., median) of the reference sample: below 80% of the median, a child is "underweight", while a child under 60% of the median is seriously underweight and has marasmus (http://www.oup.com/uk/orc/bin/9780199290970/mann_ch29f.pdf).

Biochemical methods are the essential part of the nutritional assessment. A number of biomarkers of iron status have been developed (Mei et al. 2005; Beard et al. 2006, 2007; Yang et al. 2008; Ayoya et al. 2010; Thurnham et al. 2010). Of these, the hemoglobin (Hb) concentration in the blood is commonly used to estimate the prevalence of iron deficiency. However, the Hb concentration is affected by factors besides the iron status, such as malaria, other systemic infections, hemoglobinopathies, and other micronutrient deficiencies (Yang et al. 2008). Diagnosing the iron deficiency among infants is a challenge, which may require additional measurements, besides Hb, such as measurement of ferritin, soluble transferring receptor, and the protoporphyrin levels (Beard et al. 2007). Most recent studies revealed that either plasma ferritin concentration (Yang et al. 2008) or the measurement of both

serum α_1-acid glycoprotein (AGP) and serum C-reactive protein (CRP) (Ayoya et al. 2010) may provide better indication of the iron deficiency. However, it should be noted that the ferritin increases with infection; hence, the risk of underestimating the iron deficiency. Thurnham et al. (2010) therefore suggest measuring both acute-phase proteins (APP) and CRP to estimate the full effect of inflammation that can be used to correct ferritin concentrations. Lowe et al. (2009) assessed the usefulness of a number of potential biomarkers of the zinc status to conclude that plasma, urinary, and hair zinc in healthy individuals are the reliable biomarkers of zinc status in humans. Wessells et al. (2010) also confirmed the usefulness of measuring plasma Zn concentration to monitor compliance with, and possibly effectiveness of, Zn supplementation programs. The plasma Zn concentration in their study increases within 2–5 days of starting each of the doses of Zn supplements (10 and 20 mg d^{-1} Zn), remains elevated during the period of supplementation, and declines to baseline concentrations within 14 days of discontinuing supplementation. For the vitamin A deficiency, plasma β-carotene, which indicates reduced intake and plasma retinol that indicate impaired function or cell depletion have been suggested for an early diagnosis of the vitamin A deficiency (http://www.oup.com/uk/orc/bin/9780199290970/mann_ch29f.pdf).

Blood messenger ribonucleic acid (mRNA) biomarkers for individualized disease prediction and diagnosis are an exciting area in medicine, which offer early, and more accurate prediction and diagnosis of disease and disease progression, and thus the ability to identify individuals at risk. The mRNA biomarkers in nutrition have potential application to diagnose individuals/population suffering from nutritional deficiencies. For example, the changes in white cell metallothionein mRNA were found to correlate with the changes in zinc intake in human subjects (Cao and Cousins 2000), while the microarray analysis found decreased levels of mRNA for a zinc influx transporter in women supplemented with zinc (Andree et al. 2004). More recently, Sunde (2010) demonstrated that selenoprotein-H and selenoprotein-W as well as glutathione peroxidase-1 (Gpx1) mRNA are highly down regulated in the selenium (Se) deficiency in rat liver, and the minimum dietary Se requirement based on these biomarkers is 0.06–0.07 μg Se g^{-1}, which is similar to those determined by using conventional biomarkers. Clearly, more research is needed to develop mRNA biomarker panels for all nutrients that will discriminate between deficient, marginal, adequate, and supernutritional individuals and populations, and differentiate between individuals who could benefit or be adversely affected by nutrient supplementation (Sunde 2010).

In conclusion anthropometry and biochemical tests have been proposed to diagnose micronutrient malnutrition. Hb concentration in the blood is the most commonly used method to assess iron deficiency; however, several factors including iron status and inflammation in the body have potential to impact Hb concentration. It is therefore recommended that either plasma ferritin concentration or the measurement of both serum AGP and CRP may provide better indication of the iron deficiency. The zinc concentration in blood plasma, urine, or hair is reliable biomarker, while the plasma β-carotene and plasma retinol is used to diagnose vitamin A deficiency. Use of mRNA as biomarker is an emerging area and needs further investigation to develop mRNA biomarker panels for assessing the nutritional status of populations or individuals.

III. MICRONUTRIENT BIOAVAILABILITY

A. Models and Assays to Access Nutrients Bioavailability and Absorption

1. Models and Assays. Both *in vitro* and *in vivo* techniques have been used to assess bioavailability of minerals and vitamins, with both having their own strength and drawbacks. The *in vitro* technique is designed to mimic the human digestive system, particularly conditions in the stomach and small intestine. One well-known model uses cultured human Caco-2 cells, to evaluate the digestibility and bioaccessibility of nutrients. Caco-2 cells are a human intestinal cell line originally derived from a colon adenocarcinoma to use as surrogate for enterocytes of the small intestine, which researchers have used to assess the nutrients metabolism, transport, and absorption of various nutrients (Alvarez-Hernandez et al. 1991; Glahn et al. 1996, 1998a,b; Yun et al. 2004). The Caco-2 cell model is rapid, cost effective, and compares well with human studies ($r = >0.90$) over a range of iron bioavailability for a known promoter or inhibitor (Au and Reddy 2000; Yun et al. 2004). The Caco-2 cell culture model has been used to compare iron bioavailability in common bean, maize, and rice to select those with high iron bioavailability (see Section III.A.2). Similar studies using *in vivo* models with laboratory animals are likely to cost many times a Caco-2 cell study and are often questioned given the validity of extrapolating from animals to humans (Reddy and Cook 1991). This method (Caco-2 cell culture) also allows researchers to study

interactions between minerals such as Fe and enhancer or inhibitors affecting mineral bioavailability in food (Monsen et al. 1978; Yeung et al. 2003). Another advantage of Caco-2 cell model is that bioavailability can be determined even when composition data of a given food is either inaccurate or not available.

Thus, the Caco-2 cell culture model represents a useful tool for initial screening and should be complemented with *in vivo* studies that will remain as the criterion standard for bioaccessibility of nutrients and bioactive compounds (Fernández-García et al. 2009). Other *in vitro* methods include solubility and dialyzability that are not useful predictors of iron absorption in comparison to Caco-2 cell model, which provide the most useful *in vitro* experimental approach for studying iron availability from food digests to predict about the iron bioavailability *in vivo*. However, further developments are required to optimize and standardize methodologies between different laboratories, including cell type and passage number, cell culture conditions, use of dialysis membranes for food digests that contain ferritin and other large-molecular weight iron complexes, time of exposure to food digest, harvesting of cells, and the use of reference standards so that direct inter-laboratory comparisons can be made between different food substrates (Fairweather-Tait et al. 2005, 2007).

An *in vivo* method requires various types of test animals to access bioavailability of nutrients and nutrient precursors, nutrient × nutrient interactions, nutrient tolerances, and toxicities (Baker 2008). The two strategies in *in vivo* method include balance studies and tissue concentration, which allow determination of the absorbed amount of nutrients, bioactive compounds, or their metabolites. Balance studies determine the difference between the fed and excreted amounts of the nutrient or bioactive compound. The tissue concentration consists of monitoring the increase in plasma/serum concentration of the nutrient or bioactive compound. These approaches have been applied either with experimental animal or human subjects to determine absorption of carbohydrates, minerals, vitamins, phytochemicals, and others (Fernández-García et al. 2009).

The most frequently used animals for nutrition research are chicken, mouse, rat, gerbil, preruminant calf, ferret, nonhuman primate, pig or piglets, hamster, and dog (Lee et al. 1999), which have been valuable in advancing our knowledge of nutrition, with many exhibiting well-documented differences versus humans in how they use, metabolize, and excrete nutrients (Lee et al. 1999; Baker 2008). However, because of species differences it is important to choose the right animal model for

prediction of what might happen in humans. Other potential factors influencing the choice of animal model include the availability of facilities and cost of the experiments to be performed. Choosing the most appropriate model for a study requires careful consideration. No one animal model completely mimics human absorption and metabolism of nutrients; thus, the best model must be chosen with consideration of the specific application being studied, characteristics of the model, and the available resources and facilities.

2. Nutrients Bioavailability, Absorption, and Metabolism. The key to nutritional efficiency is bioavailability. It refers to the fraction of the nutrients or bioactive compounds available for use in physiologic function or for tissue storage. Both food and host factors influence bioavailability of the nutrients. Bioavailability of nutrients in humans is determined by a sequential series of events, which include (1) digestion and release of elements from food matrix into the lumen of gastrointestinal (GI) tract (*availability*), (2) transport into intestinal enterocytes (*uptake*), (3) efflux across the basolateral membrane of enterocytes into the circulation (*absorption*), (4) retention, or endogenous excretion in urine and feces (*retention*), (5) transport to tissues for use in normal body functions (*utilization*), and (6) transport to storage sites (*body stores*) (Fairweather-Tait et al. 2005). Furthermore, Fernández-García et al. (2009) defined bioavailability as a sum of bioaccessibility and bioactivity and defined bioaccessibility as the fraction of a compound that is released from its matrix in the GI tract and thus becomes available for intestinal absorption. Bioaccessibility includes the entire sequence of events that take place during the digestive transformation of food into materials that can be assimilated by the body, the absorption/assimilation into the cells of the intestinal epithelium, and lastly, the presystemic metabolism. Bioactivity includes events linked to how the bioactive compound when and how it is transported and reaches the target tissue, how it interacts with biomolecules, the metabolism or biotransformation that it may undergo, and the generation of biomarker and the physiologic response it causes. There are different analytical approaches and models, which have been advocated to measure bioaccessibility of nutrients and bioactive compounds, and these are described below with respect to iron, zinc, and β-carotene utilization by humans.

3. Iron and Zinc. Traditionally rats have been suggested as the animal model for performing nutrition studies; however, the rat model has a

number of limitations that makes extrapolation back to a human situation questionable, including significantly different food intake and energy expenditure for body size, a different life-span and body proportion, differences in intestinal morphology and enteric microbiota, as well as other distinct physiological differences (Gregor 1992). Another major problem with using rat models for mineral studies is their propensity for practicing coprophagy, which may have dramatic impact on nutritional study.

Pigs share many similarities with humans making them a valuable experimental model for nutrient bioavailability and absorption. The difference between human and pig is in their intestine lengths and spatial arrangements of the intestines within the abdominal cavity. The small intestine of adult pig is around 15–22 m, whereas the large intestine has an average length of 4–6 m. In contrast, the small intestine of a human adult averages around 5.5–7 m, whereas the large intestine is around 1.5 m. In spite of these differences, the porcine digestive and metabolic processes function in much the same way as those of humans, and digest transit times are also similar between the two species. The intestinal villus structure and component epithelial cells are also very much alike, which makes pigs an ideal model for human nutritional studies to investigate bioavailability and digestibility of various dietary factors in gastrointestinal compartments (Patterson et al. 2008).

Chickens have a shorter intestinal tract, 2.2 m, relative to humans (Sturkie 2000). The duodenum in chicken is the primary iron absorption site, a feature similar to humans (Sturkie 2000). Chickens could be a relevant model as a source of tissue for *in vitro* iron bioavailability studies, *in vivo* feeding trials, or both. Recently, Tako et al. (2009b) evaluated broiler chickens as a model for assessment of iron bioavailability using iron-deficient and -nondeficient (control) birds and a unique duodenal loop technique for direct measurement of iron absorption. They detected higher iron absorption in the iron-deficient birds. In addition, expression of proteins involved in iron uptake and transfer were elevated in the low iron group, and concluded that this model exhibits the appropriate responses to Fe deficiency and has potential to serve as a model for Fe bioavailability.

Haas et al. (2005) used poultry model in plant breeding to select for traits that enhance the nutritional quality of crops by increasing iron concentration or bioavailability or both. The poultry model therefore could serve as an intermediate test of *in vivo* iron bioavailability in preparation for subsequent human studies.

Several reports indicate phytic/zinc (P/Z) molar ratio a poor indicator of zinc bioavailability as it does not take into account the aggravating

effects of calcium on zinc absorption in phytic acid containing diets (Forbes et al. 1983, 1984). However, phytate × calcium/zinc ratio in most of the soybean-based processed foods is a better predictor of zinc bioavailability than the phytate/zinc ratio (Fordyce et al. 1987). Further, Miller et al. (2007) developed a mathematical model of zinc absorption as a function of dietary zinc and phytate, which they tested on select existing data sets to find a good fit ($R^2 = 82\%$) that demonstrate the validity of the model to study zinc nutrition and metabolism and estimate dietary zinc requirements in varied populations. Similarly, the phytate/iron molar ratio has been suggested as an indicator of iron bioavailability in beans (Ariza-Nieto et al. 2007; Pachón et al. 2009).

4. β-Carotene. Foods containing provitamin A carotenoids (β-carotene, β-cryptoxanthin, and α-carotene) are the primary source of vitamin A. Mechanisms regulating bioavailability and bioconversion of carotenoids include (1) the release of carotenoids from the food matrix, (2) the solubilization of carotenoids into micelles, (3) the uptake of carotenoids into intestinal mucosal cells, (4) the absorption of intact carotenoids, (5) the cleavage of provitamin A carotenoids within the enterocytes or within other tissues to yield vitamin A, and (6) the tissue distribution, metabolism, and recycling of carotenoids (Castenmiller and West 1998). An ideal model for carotenoids research should be the one that (1) absorb a variety of carotenoids intact at the physiologic levels in the same way as with humans, (2) have carotenoids distribution in tissues similar to that of humans, and (3) represent an appropriate model for the disease state of interest as many diseases in humans have been diagnosed due to vitamin deficiency. Like humans, gerbil, ferrets, and preruminant calves all absorb β-carotene intact, but only gerbils and calves convert β-carotene to vitamin A with efficiency similar to that of humans (Lee et al. 1998, 1999; Howe and Tanumihardjo 2006). The gerbils are small, easily maintained in large numbers, and readily available. They are also an established model for cholesterol and lipid metabolism because their serum lipid profile responds to dietary changes similarly to humans (Lee et al. 1999).

The *in vitro* Caco-2 model have also been suggested to measure bioavailability and absorption of carotenoids including β-carotene as a rapid and cost-effective model for assessing bioavailability of carotenoids from meals (Garrett et al. 1999; Liu et al. 2004), which varies widely both for different carotenoids (Reboul et al. 2006; Granado-Lorencio et al. 2007; Kean et al. 2008; Failla et al. 2009; O'Sullivan et al. 2010). Alternately, the TNO gastrointestinal tract model (TIM) is a dynamic computer-controlled *in vitro* system that closely mimics the

physiological processes occurring within the lumen of the stomach and small intestine of humans (Minekus et al. 1995). The TIM system reproduces (1) three compartments of the human small intestine, (2) chime transit from one digestive compartment to the next, (3) pH change during the gastric digestion and from the duodenum to ileum, (4) sequential arrival of digestive secretions, and (5) passive absorption of small molecules and water. This model has shown its usefulness in studying the digestive stability of carotenoids from different food matrices throughout the gastrointestinal tract (Blanquet-Diot et al. 2009). Combining the TIM system with Caco-2 cells, Déat et al. (2009) found the potential applicability and predictive value of this *in vitro* approach to access the bioavailability of bioactive compounds from food or supplements.

B. Factors Influencing Nutrients Bioavailability

1. Enhancers/Inhibitors. There are two types of dietary iron: non-heme iron that is present in both plant foods and animal tissues, and heme iron that comes from hemoglobin and myoglobin in animal source foods. The heme iron is more readily and rapidly absorbed than non-heme iron. Other factors that influence the iron bioavailability could be broadly grouped as either enhancer or inhibitor of nutrients. The former includes ascorbic acid, meat, fish, and poultry, while the latter phytate, polyphenols, calcium, some plant proteins, wheat bran, and fiber (Kalgaonkar and Lönnerdal 2008; Hurrell and Egli 2010; Petry et al. 2010). Variation in seed color impacts its quality. Lung'aho and Glahn (2010) investigated the effect of seed coat color on iron bioavailability from a Tanzanian complementary food mixture. They detected that white-seeded beans had a significantly higher amount of ferritin but lower amount of tannins when compared to all other porridge ingredients including the red-seeded beans, suggesting that substitution of complementary food ingredients with high antinutrient concentrations (such as colored seeds) with those that have lower antinutrient concentrations (such as white seeds) may improve bioavailability from complementary food home recipes.

Polyphenols in foods may chelate dietary Fe and lower its bioavailability. Red beans have higher polyphenols than white-seeded beans. Hu et al. (2006) compared iron bioavailability from colored beans (white, red, pinto, and black beans) using an *in vitro* digestion Caco-2 cell culture model, while others (Tan et al. 2008; Tako et al. 2009a) used both *in vitro* and *in vivo* (pigs) to compare Fe bioavailability between

colored beans. The former detected 10-fold higher bioavailable Fe in white beans, suggesting that color-importing polyphenols inhibit iron absorption, while the latter found no difference in bioavailable Fe between red and white beans, which is because pigs seem able to adapt to the inhibitory effects of polyphenols on Fe absorption by increasing the secretion of protective protein-rich proteins in their saliva. More recently, Petry et al. (2010) studied the influence of bean polyphenols relative to phytic acid on iron bioavailability in humans (young women). This study revealed lower iron absorption by 14% with 50 mg polyphenol ($P < 0.05$) and by 45% with 200 mg polyphenols ($P < 0.001$). The mean iron absorption from whole bean porridge was 2.5%. Polyphenol and phytic acid removal increased absorption 2.6-fold ($P < 0.001$) and removal of polyphenol from dephytinized porridge doubled iron absorption ($P < 0.001$). Dephytinization did not increase iron absorption in the presence of polyphenol, but in their absence, absorption increased 3.4-fold ($P < 0.001$), which indicate that both polyphenol and phytic acid should be reduced to enhance iron bioavailability in bean. The lowering only one inhibitor will have a modest influence on iron absorption.

Both garlic and onion are rich sources of dietary sulfur-containing amino acids. Gautam et al. (2010a) reported the enhancing effect of these two species on iron bioaccessibility in cereals (9.4%–65.9% increase) and legumes (9.9%–73.3% increase) in both raw and cooked conditions. These two species similarly enhance the bioaccessibility of zinc from the food grains, the increase ranges from 10% to 159% in cereals and from 10% to 50% in pulses. This novel information has the potential application in evolving a food-based strategy to improve the bioavailability of minerals and hence contribute to the human health benefit. Malting generally improves the nutrient content and digestibility of foods. Platel et al. (2010) reported increased effect of malting on bioaccessibility of iron by >threefold in finger millet and by >twofold in wheat, whereas no such effect seen in barley; however, malting increased bioaccessibility of zinc to the extent of 234% in wheat and 100% in barley. In contrast, malting reduced the bioaccessibility of zinc in finger millet.

Green leafy vegetables and orange/yellow colored fruits are rich source of carotenoids, and a beneficial effect of vitamin A and β-carotene on enhanced nonheme irons absorption has been reported (García-Casal et al. 1998; Layrisse et al. 2000). The presence of vitamin A increases iron absorption up to 3 times for rice, 2.4 times for wheat, and 1.8 times for maize, while β-carotene increases iron absorption almost 3 times, showing that both compounds prevent the inhibitory

effect of phytates on iron absorption (Layrisse et al. 2000). More recently, Gautam et al. (2010b) evaluated β-carotene-rich vegetables on bioaccessibility of iron and zinc in cereals and legumes-based foods. Addition of carrot or amaranth significantly enhanced the bioaccessibility of iron and zinc from the food grains, the percent increase being 13.8–86.2 in the case of carrot and 11–193 in the case of amaranth. Pure β-carotene added at an equivalent level also enhanced the bioaccessibility of iron by 19.6%–102% and zinc by 16.5%–118% from the cereals examined. This positive effect of β-carotene-rich sources on the bioaccessibility of either iron or zinc is generally greater in the cooked grains than the raw grains.

The iron status of the individual, food additives, such as erythorbic acid, and other host-related factors, such as obesity, play a key role in iron bioavailability (Hurrell and Egli 2010). Some unresolved issues that require further investigation include the mechanism by which calcium inhibits iron absorption, the nature of meat factor, the role of inulins on microflora, the influence of vitamin A and carotenoids, and nondigestible carbohydrates on iron bioavailability (Hurrell and Egli 2010). An earlier report revealed the beneficial effect of vitamin A and carotenoids on iron bioavailability (García-Casal et al. 1998).

2. Production Environment, Postharvest and Storage Conditions, and Food Processing and Preparation. Burt et al. (2010) studied the effect of postharvest factors on carotenoids concentration and composition in high-carotenoid maize kernels. A preliminary trial using room temperature drying indicated that while carotenoids profiles remain stable during storage, carotenoids levels decrease significantly from initial levels between 3 and 6 months of storage, but then remain stable for another year. Further, a more detailed study using three drying and storage regimes (freeze-drying and storage at −80°C; room temperature drying and storage; 90°C drying and room temperature storage) reveals that extreme caution is needed to maintain carotenoids levels in maize during handling and storage, but in situations where freeze-drying is not possible, high heat drying is no more detrimental than low heat drying. In OFSP, hot air cross flow drying retained significantly more provitamin A than sun drying, while no significant difference in provitamin A retention between solar and sun drying. The shape of the sweet potato pieces (chip or crimped slice) also influenced provitamin A retention during sun drying: crimped slices retained more provitamin A (Bechoff et al. 2009). Further studies on effect of drying and storage on the degradation of carotenoids in OFSP reveals that carotenoids losses during drying are low (15% or less) and carotenoids

retention is not dependent on the type of dryer (solar or sun). However, 4 months of dried sweet potato chips storage at room temperature resulted ~70% of carotenoids losses, independent on the use of opaque or transparent packaging (Bechoff et al. 2010). Furthermore, Wolbang et al. (2010) reported significant cultivar effects on β-carotene content, its bioaccessibility, and antioxidant properties in melons. Sowing time significantly affected β-carotene contents, and antioxidant potentials, but these were highly cultivar dependent, with season having no effect. Postharvest storage over 4 weeks at 7°C resulted in losses of antioxidant potential and β-carotene concentration independent of cultivar. Furthermore, genotypic differences in maturity among maize hybrids were associated with β-carotene concentration in grain, thus, more research is warranted to study the effects of maturity, environment, and timing of harvest on provitamin A concentrations in grains (Pixley et al. 2011a).

Several traditional food processing and preparation methods including thermal processing, mechanical processing, soaking, fermentation, and germination/malting are reported to significantly enhance the bioavailability of micronutrients in plant-based diets (Erdman and Pneros-Schneier 1994; Eyzaguirre et al. 2006; Hotz and Gibson 2007; Hemalatha et al. 2007a,b). Processing has the potential to impact bioavailability of carotenoids (van het Hof et al. 1999; Reboul et al. 2006; Mamatha et al. 2010). Preparation methods (boiling, roasting, temperature and the duration of roasting) also impact both the retention and bioavailability of β-carotene in cassava (Thakkar et al. 2009); however, steaming/boiling of cassava resulted significantly lower β-carotene losses than either solar drying or open-air sun drying OFSP slices (Bengtsson et al. 2008). Fermented maize flour is used to prepare porridges and a variety of other staple foods in West Africa. Fermentation provides an optimal pH for enzymatic degradation of phytate, which may increase the amount of soluble calcium, iron, and zinc, and also contributes to the safety, shelf-life, and acceptability of maize-based foods (Blandino et al. 2003). The fermentation does not adversely affect the retention of provitamin A carotenoids in porridges prepared with high β-carotene maize (Li et al. 2007). Veda et al. (2008) reported the beneficial effect of food acidulants (amchur, lime, tamarind, and kokum) and antioxidant spices (turmeric and onion) on the bioaccessibility of β-carotene in fleshy and leafy vegetables. Amchur and lime enhance the bioaccessibility of β-carotene, both in raw and heat-processed vegetables, with more pronounce effect of lime juice than amchur. In contrast, turmeric significantly enhance the bioaccessibility of β-carotene, especially when heat-processed vegetables. Onion enhances the bioaccessibility of β-carotene from the pressure-cooked carrot and amaranth leaf and from open-pan-boiled

pumpkin and fenugreek leaf. In addition, lime juice, turmeric, and onion minimized the loss of β-carotene during heat processing of the vegetables.

The presence of other carotenoids in a meal may also adversely affect the bioavailability of provitamin A carotenoids (van den Berg 1999). The absorption of carotenoids from the meal undergo a series of processes that include its initial transfer from food matrix to oil droplets in the gastrointestinal lumen, partitioning of the pigments into mixed micelles for delivery to absorptive epithelial cells during the small intestinal phase of digestion, and the uptake and incorporation of the pigments into chylomicrons secreted into lymph, and *in vitro* studies support the interactions between carotenoids during preabsorptive processes (Borel et al. 1996; van den Berg and van Vilet 1998; Tyssandier et al. 2001). More recent results revealed that bioaccessibility of provitamin A carotenoids is minimally or not affected by other carotenoids (Davis et al. 2008; Thakkar and Failla 2008).

The preformed vitamin A is readily available from foods, while carotenoids are much more difficult to assimilate. A number of factors have been identified that either enhance or hinder the bioavailability of carotenoids and the bioconversion of food provitamin A carotenoids to vitamin A in humans. These include species of carotenoids, molecular linkage, amount in the meal, matrix (food) properties, effectors, nutrient status, genetic, host specificity, and interactions between factors, often abbreviated as SLAMENGHI (Tanumihardjo 2002; Reboul et al. 2006; Tang 2010). Conversion of β-carotene (provitamin A) into vitamin A in humans takes place predominantly in the intestine.

3. Provitamin A (β-Carotene) Conversion to Retinol (Vitamin A). Wide variation in carotenoid to vitamin A conversion factors, ranging from 3.6:1 to 28:1 by weight, not only between studies but also between individuals in a particular study have been reported, which show that the vitamin A value of individual plant foods rich in provitamin A carotenoids may vary significantly, thus, meriting further investigation for the development of dietary guidelines to combat vitamin A deficiency worldwide (Tang 2010). Furthermore, Li et al. (2010b) quantified the vitamin A equivalence in the β-carotene-biofortified maize porridge consumed by women and found that on average 6.48 ± 3.51 μg of the β-carotene in β-carotene-biofortified maize porridge and 2.34 ± 1.61 μg of the β-carotene in the reference dose were each equivalent to 1 μg of retinol, which suggest that β-carotene in biofortified maize has good bioavailability as a plant source of vitamin A. Similarly, β-carotene derived from 'Golden Rice' is effectively converted to vitamin A (0.24–0.94 mg retinol) in humans. Thus, the conversion factor of Golden Rice β-carotene

to retinol is (3.8 ± 1.7)–1 with a range of (1.9–6.4)–1 by weight, or (2.0 ± 0.9)–1 with a range of (1.0–3.4)–1 by moles (Tang et al. 2009).

C. Efficacy of Biofortified Crops on Human Health

Selective breeding has resulted into the development of a number of nutritionally enhanced advanced lines and hybrids such as quality protein maize (QPM) (Atlin et al. 2011); mineral-dense (Fe and Zn) common bean, maize, pearl millet, rice, and wheat; β-carotene-rich sweet potato, cassava, maize, and rice, with a few of these already released for cultivation in some countries (see Section VI.E). To date, only few studies have been conducted to assess the bioavailability to humans of micronutrients from these enriched lines/hybrids. A 9-month human efficacy study, involving seed Fe-dense rice line IR68144 and human subjects in the Philippines (192 religious sisters), revealed that the consumption of biofortified rice without any other changes in diet is efficacious (increased body iron by 20%) in improving iron stores of women with iron-poor diets in the developing world (Haas et al. 2005; http://nutrition.org/cgi/content/full/135/12/2823). Rosado et al. (2009) compared the intake and absorption of Zn in adult women who consumed tortillas made either from biofortified or nonbiofortified wheat. The study revealed higher Zn intake from biofortified wheat (5.7 mg g^{-1} at 95% extraction and 2.7 mg g^{-1} at 80% extraction) compared to that with the corresponding control wheat, while the Zn absorption from biofortified wheat meals at both extraction level was comparable (~2 mg g^{-1}), which was 0.5 mg day^{-1} higher than that from the corresponding control wheat, demonstrating that valuable increases in Zn absorption can be achieved from biofortified wheat. Several low phytate (lp) mutants have been reported in barley, common bean, maize, rice, soybean, and wheat (see Section V.A). In a study on the effects of Zn absorption among Guatemalan school children fed with lp maize, its corresponding wild type (WT) maize, or local maize, Mazariegos et al. (2006) found variable phytate (lp: 1,536 mg d^{-1}, WT: 2,056 d^{-1}, local: 2,253 d^{-1}) and zinc (lp: 8.6 mg d^{-1}, WT: 8.1 mg d^{-1}, local: 9.7 mg d^{-1}) intakes, and dietary phytate/Zn molar ratio (lp: 18, WT:26, and local: 23), the corresponding fractional absorptions of zinc (lp: 0.32, WT: 0.28, local: 0.29) and total absorbed zinc (lp: 2.72 mg d^{-1}, WT: 2.30 mg d^{-1}, local: 2.78 mg d^{-1}) were similar between the maize groups, which indicates that lp maize did not show an altered efficiency of zinc absorption in this population.

β-carotene-rich OFSP is an excellent source of provitamin A. In a study conducted on primary school children in south Africa, van Jaarsveld et al. (2005) found that the consumption of OFSP improves vitamin A status,

which can be used as a viable long-term food-based strategy for controlling vitamin A deficiency in children in developing countries. Further, Low et al. (2007) found that integrated promotion of OFSP contributes to increases in vitamin A intake and serum retinol concentrations in young children in rural Mozambique. The vitamin intake in intervention children was much more than those of control children. A high β-carotene (15 μg^{-1}) temperate hybrid, CI7 × DEexp, is currently being investigated for its efficacy in human and animal (Yan et al. 2010).

Using proxy measures and recipe prepared from nutritionally enhanced bean (NEB) that has more Fe and Zn than conventional bean (CB) and nutritionally enhanced maize (NEM) that has more tryptophan and lysine than conventional maize (CM), Pachón et al. (2009) detected similar Fe in the cooked NEB and CB and in NEM and CM; similar *in vitro* digestibility of Fe in cooked NEB and CB but greater in NEM than CM; higher Zn in uncooked and cooked NEB than in the CB but lower bioavailability of Zn due to higher phytate:Zn molar ratios in the cooked NEB and CB. Further, they detected no such differences in Zn concentration or phytate/Zn molar ratios in the maize recipe. The *in vitro* protein digestibility was comparable for NEM and CM, but was higher for NEB than for CB, which reveals that nutritionally enhanced crops can improve human nutrition if it translates into more nutrients absorbed and utilized by the body (Pachón et al. 2009). Using meta-analysis and community-based approach, Gunratna et al. (2010) studied the nutritional impact of QPM, which revealed that consumption of QPM instead of conventional maize led to a 12% increase in the rate of growth in weight and a 9% increase in the rate of growth in height in infants and young children with mild-to-moderate undernutrition from populations, with maize as staple food. In another study on young children in the Ethiopian highlands, the inclusion of QPM in children's diet could reduce or prevent growth faltering and may in some cases support catch-up growth in weight (Akalu et al. 2010). Clearly, more such studies are needed to assess the efficacy of biofortified crops on human health.

In summary, the Caco-2 model is the most frequently used in the *in vitro* assay to assess the bioavailability of micronutrients including Fe, Zn, and β-carotene. It is a useful tool for initial screening; however, should be complemented with the *in vivo* studies. No one animal model is perfect to completely mimic human absorption and metabolism of nutrients; however, pigs share many similarities with humans making them a valuable *in vivo* model for Fe and Zn bioavailability and absorption. For β-carotene, gerbils and calves are appropriate models as both convert β-carotene to vitamin A with efficiency similar to that of humans. The food-based diets contain several enhancers/inhibitors that impact

nutrients bioavailability and absorption. Production environments, post-harvest drying/storage, and food processing and preparation methods have potential to adversely impact loss (degrade) and/or bioavailability of β-carotene. The carotenoids as a whole promote Fe bioavailability. The preliminary studies involving biofortified crops revealed that consumption of biofortified food is efficacious to improving Fe and Zn levels on humans.

IV. PHENOTYPIC SCREENS

A. Methodology and Approaches to Screen for Seed Iron, Zinc, Phytate, and β-Carotene

To select or breed crop cultivars denser in seed iron, zinc, or β-carotene, it is important to use standardized methodologies for screening the materials. Such an approach is a prerequisite for comparing results across various locations and sites. It is also important to make sure that the medium of growth (generally soil in a field experiment) is supplied with known amounts of all nutrients including Fe and Zn for growth, development, and seed production as interactions between nutrient uptake and seed content are well-known. Multisite testing will ensure expression of genetic differences, if any; in micronutrients and help determine the heritability and genotype × environment interaction for each element or vitamin. In addition, soil and environmental factors–especially soil water regime and climatic factors such as temperature–also influence yield and the seed quality relative to Fe and Zn composition of a crop (Stewart et al. 2005). Since seed Fe and Zn are liable to contamination during harvest and preparation of the samples (grinding) for analysis in the laboratory using routine methods, extra precautions are needed to avoid contamination with Fe and Zn during these operations (Mills and Jones, 1996). Grinding with Teflon chambers or at least stainless steel or fiber mills is recommended (Stangoulis and Sison 2008; Blair et al. 2009a).

In the literature, results on the mineral composition including Fe, Zn, and β-carotene of seeds of various crops are reported as concentration (Velu et al. 2006; Graham et al. 2007; Cakmak 2008; Demirkiran 2009; Tiwari et al. 2009) that refers to mass or molar ratio such as mg or moles kg^{-1} seed. But sometimes, the quantity of nutrients in plant parts or the whole plant is expressed as uptake or content (expressed as μg $seed^{-1}$ or organ). The use of the term concentration and content should be precise so as to refer to the appropriate aspect of nutrient physiology. Uptake and content of nutrients are influenced by dilution; and the

concentration decreases due to increased dry matter and increases due to a loss of plant dry matter (Jarrell and Beverly 1981). Seed mineral content reflects the supply of that nutrient to the individual growing seedling and is therefore of agronomic importance, while concentration is more important in overall nutritional terms.

Several authors have reported on the yield dilution of seed Zn and Fe in various crops including wheat and maize (Oikeh et al. 2003b; Lyons et al. 2005; Pleijel and Danielsson 2009). It is thus suggested that the term concentration (mg kg^{-1} seed) as defined above should be preferred to express minerals in seed of various crops. The content (μg $seed^{-1}$) of a mineral in seed is influenced by growing conditions that affect seed size. For example, with increased seed size, the amount of the mineral will be diluted compared to low seed size if the amount of mineral nutrient is fixed. Blair et al. (2011) pointed this out in a comparison of content versus concentration among various legumes where model legumes have tended to be small seeded while crop legumes are large seeded, especially as with Andean common beans. This is especially true if there is variability among seed tissues such as the seed coat, embryo, or cotyledonary tissue, unless compensation between these tissues occurs (Ariza-Nieto et al. 2007). For example, micronutrient levels in the embryo and seed coat are much higher than in the endosperm. Hence, seed shriveling, wrinkling, and weathering can result in elevated micronutrient concentrations–the "concentration" effect–given that the seed coat-to-endosperm ratio is much higher than in normally developed grains (Cakmak et al. 2000; Imtiaz et al. 2003). Assessing the correlation between micronutrient concentration and content can help to determine whether seed size and shriveling affect micronutrient concentration of a given sample of genotypes (Pfeiffer and McClafferty 2007). More recently, Velu et al. (2011) detected highly significant correlation between the concentration and content of grain Fe ($r = 0.45$, $P < 0.01$) and Zn ($r = 0.65$, $P < 0.01$), which suggest that higher grain Fe and Zn concentrations are not necessarily related to small grain size or weight in wheat.

The atomic absorption spectrophotometer (AAS) method has been suggested for routine estimation of Fe and Zn in seeds (Sahrawat et al. 2002; Blair et al. 2009b, 2010a,b,2011). An excellent review on the role of atomic spectrometry in plant science has been published elsewhere, and it is important to recognize recent developments in multielemental and speciation analyses in plants with the resulting functional roles of different elements in plant science (Husted et al. 2011). X-ray fluorescence spectrometry is another method that allows identifying a wide range of micronutrients including elements such as P, which is

indicative of the antinutrient phytate. Inductively coupled plasma (ICP) analysis is more of a gold standard but is expensive and therefore of less utility in breeding especially as it is usually highly correlated with AAS readings (Blair et al. 2009b, 2010c) and is the current method of choice to detect elements such as aluminum, which has been proposed as an indicator of iron contamination (Stangoulis and Sison 2008).

Lorenz et al. (2007) developed a rapid and inexpensive method for measuring phytate and inorganic phosphorus (Pi) concentrations in maize, which provides adequate precision and simplicity to deal with large number of breeder's samples for estimating phytate and Pi levels simultaneously. Estimates obtained from this technique match closely with those obtained from ion exchange methods, and the repeatability of the values across fields suggests that the protocol can be used to make heritable measurements for both phytate and Pi. Near infrared reflectance spectroscopy (NIRS) is another possibility (Blair et al. 2008, 2009a).

The β-carotene content in seeds of crops is generally analyzed using high performance liquid chromatography (HPLC) as this method is more precise than the colorimetric assays (Bhaskarachary et al. 1995; Rodriguez-Amaya and Kimura 2004). Further, Hulshof et al. (2007) developed a fast screening (in comparison to HPLC) of maize seeds, which allows distinction between lines the low, medium, and high levels of provitamin A carotenoids by semiquantitative analysis without the need of a full HPLC analysis of all samples, and hence reduces the cost of analysis.

B. Screening Under Optimal or Nutrient-Deficient Conditions

Germplasm pools are ideal biological resources to mine allelic variation for beneficial traits including seed micronutrients. Rapid and cost-effective phenotypic screens significantly impact the potential to developing seed mineral-dense cultivars. Questions are often asked whether to screen germplasm/breeding populations under optimal soil conditions (not deficient in micronutrients), pot culture using mineral-deficient soils or in hydroponics system, or under natural occurring sites deficient in micronutrients. It is feasible to identify seed mineral-dense genotypes by evaluating germplasm/advanced breeding lines under optimal soil conditions (see Section V.A); however, it is quite possible that such germplasm may not show any advantage when grown under mineral-deficient soils. Moreover, research to date reveals strong location effects on micronutrients than genotype by environment interaction effects (see Section VI.B). In particular, seed Zn and, less so, Fe is influenced by environmental factors, as indicated also by lower heritability of these minerals when compared with provitamins A (Pfeiffer

and McClafferty 2007). An alternative approach would be to identify natural occurring sites deficient in micronutrients for germplasm evaluation to identify genotypes efficient in mineral acquisition and/or remobilization from shoot to seeds.

Mapping soils in geographic regions will go a long way to sustain breeding programs for developing seed micronutrient-dense food crops. Some efforts have been made to map geographic regions deficient in soil micronutrients, for example, (macro- and micro-) nutrient-deficient soils in India (Singh 2008) or zinc-deficient soils in Turkey (Cakmak et al. 1996). More such efforts are needed to identify a representative set of regions to initiate targeted breeding programs for developing micronutrient-dense and micronutrient-deficiency adapted food crops. An alternative to this is to (1) initially screen germplasm under optimal soil conditions to identify genotypes containing high seed micronutrients, (2) use pot-screening technique (with micronutrient-deficient soils) to characterize seed micronutrient-dense germplasm for nutrient use efficiency (acquisition and remobilization), and (3) evaluate nutrient-efficient germplasm to identify seed mineral-dense germplasm with good agronomic traits.

The link between the soil nutrient deficiency adaptation and seed loading of a micronutrient must be further studied, so that such germplasm may be intercrossed among themselves to select for multiple traits related to increased nutrient uptake and remobilization (from shoot to developing seeds) or crossed with locally adapted cultivars to transfer these traits into adapted genetic backgrounds. Ideally, such breeding populations should be evaluated under micronutrient-deficient soils. Alternatively, such populations may be advanced under optimal soil conditions by single-seed descent technique, and only at a later stage the advanced breeding lines should be evaluated in micronutrient-deficient soils for agronomic traits including seed micronutrients per se to select those that performed best under such soils. Genotype ranking could be used as a selection criterion to identify materials that rank similar across locations for seed micronutrients and agronomic traits, including seed yield and seed weight. Finally, the relationship of micronutrient uptake with soil pH, macronutrient fertilization, soil organic matter, and presence of other cations should be analyzed for each crop when considering a micronutrient breeding program [Centro Internacional de Agricultura Tropical (CIAT), unpublished].

C. Plant Traits Associated with Increased Acquisition of Iron and Zinc

Higher plants acquire Fe from the rhizosphere through two strategies—strategy I is employed by dicotyledonous and nongraminaceous

monocotyledonous species, while strategy II is used by graminaceous monocotyledonous species. Strategy I involves the induction of membrane-bound Fe(III)-chelate reductases that reduce Fe(III) to the more soluble form Fe(II), followed by uptake of Fe(II) via Fe(II) transporters. Strategy II involves the secretion of phytosiderophore (PS) by roots to solubilize soil Fe(III) (Marschner et al. 1986). Rice is an exception in the sense that it possesses both systems for Fe uptake, making it especially Fe efficient (Cheng et al. 2007).

Plant roots and their exudates greatly facilitate the availability of various plant nutrients by bringing them in the soluble form in the soil (Neumann and Römheld 2002; Rengel 2002; Ryan and Graham 2002; Welch and Graham 2004). Plant roots exude a range of organic compounds and inorganic ions into the rhizosphere that play a crucial role in the availability of and acquisition by plants of plant nutrients, especially Fe and Zn. Equally important are the differences in root exudation among genotypes that differ in tolerance to Fe and Zn deficiency. Genotypic differences in nutrient acquisition–an important determinant of nutrient use efficiency–are associated with root size and morphology, root physiology, increased root (adventitious) production, soil volume explored by roots, and the availability of Fe and Zn in the growing medium (because Fe and Zn deficiency in the soil induces exudation) (Rengel 2002; Lynch 2007; Widodo et al. 2010). Further, root surface area can be enhanced through mycorrhizal associations, while the root processes can affect rhizosphere pH and redox potential (Marschner et al. 1986; Garrido et al. 2006).

A lot of research has been conducted on the role of root exudates on the acquisition, translocation, and utilization of phosphorus (P) by diverse crop species (Lynch 2007). However, there is a paucity of information on the role of roots and root exudates on the acquisition of Fe and Zn by crops. The limited literature available indicates that root exudates are indeed important for the acquisition of Fe and Zn, especially by crops such as chickpea grown on calcareous and alkaline pH soils in which these nutrients are poorly available due to high pH. Moreover, genetic variability exists for Fe and Zn acquisition in chickpea on alkaline pH soils (Ali et al. 2002), which is associated with the mobilization of Fe and Zn in the rhizosphere via protons, organic acids, and phenolics in dicots and via phytosiderophores (PS) such as nicotianamine in cereals (Rengel 2002; Welch and Graham 2002; Lynch 2007).

More specifically, graminaceous plants including staple cereals such as wheat and sorghum when grown in calcareous soils with lower Fe and Zn availability release mugineic acid (MA) family of PS from their roots to enhance uptake and translocation of Fe and Zn to the leaves and

seeds (Marschner et al. 1986). Rice, wheat, maize, and sorghum are more susceptible to micronutrient deficiency than barley, with the former secreting only 2′-deoxymugineic acid (DMA), while the latter (barley) in addition to DMA, also release MA, 3-epihydroxy-2′-deoxymugineic acid (epi-HDMA), and 3-epihydroxymugineic acid (epi-HMA) under micronutrient-deficient conditions (Kobayashi et al. 2008). When investigating wheat (*Triticum aestivum*) cultivars and related nonprogenitor *Aegilops* species for release of PS *in vitro* under Fe- and Zn-sufficient and -deficient conditions, Neelam et al. (2010b) detected three to four times higher release of PS in *Aegilops* species than in wheat cultivars under both nutrient-sufficient and -deficient conditions. Furthermore, the absolute amount of Fe and Zn under both conditions was nearly three times higher in roots and shoots of *Aegilops* species than wheat cultivars. The amount of PS released was highly significantly correlated with Fe ($r = 0.94$) and Zn ($r = 0.91$) in roots. The higher amount of both Fe and Zn in *Aegilops* species compared with that of wheat cultivars under deficient conditions reveal that *Aegilops* species possess an efficient system for the uptake and translocation of these micronutrients to the leaves and ultimately to seeds.

Nozoye et al. (2011) report that the efflux of DMA, the primary phytosiderophore from rice and barley, involves the *TOM1* and *HvTOM1* genes, respectively, the missing piece in the mechanics of the Fe acquisition by graminaceous plants, which reveals that the TOM1 and HvTOM1 proteins are the phytosiderophore efflux transporters. Under conditions of iron deficiency, rice and barley roots express high levels of *TOM1* and *HvTOM1*, respectively, and the overexpression of these genes increased tolerance to iron deficiency. Further, in rice roots, the efficiency of DMA secretion is enhanced by the overexpression of *TOM1* and decreased by its repression, providing further evidence that *TOM1* encodes the efflux transporter of DMA. Furthermore, Widodo et al. (2010) showed that Zn-deficiency tolerant line RIL46 acquires Zn more efficiently and produces more root biomass than its nontolerant line IR74 at low $[Zn]_{ext}$ under field conditions. This observation they related with the maintenance of root growth and increased root exudation and uptake of Zn-ligand complexes [DMA and low molecular weight organic acids (LMWOA)] at low $[Zn]_{ext}$, which could possibly be used as potential breeding targets for enhancing Zn concentration in rice seeds (Widodo et al. 2010).

The roots of bread wheats tolerant to Zn deficiency exude more phytosiderophores than sensitive bread and durum genotypes, that is, greater tolerance to Zn deficiency among wheat genotypes is

associated with increased exudation of phytosiderophores and increased Fe uptake by roots, perhaps as a response to a decreased rate of Fe transport to the shoots (Rengel and Römheld 2000). Clearly, more emphasis should be placed toward understanding the role of various plant traits involved in the acquisition of Fe and Zn by crops.

D. Iron and Zinc Uptake, Accumulation, and Translocation to Seed and Nonseed Parts

Soil is the main source of nutrients–including Fe and Zn–for plant growth, productivity, and accumulation in the seed and nonseed parts of the plant. Even organic and mineral fertilizers (except foliar applicants) are applied to supply Fe and Zn to plants through the soil and become part of the soil before they are taken up by the growing plants and metabolized in seed and nonseed parts.

The availability of Fe and its functions in the soil are mostly based on (a) the reversible redox reactions of Fe^{2+} (ferrous, reduced form of Fe) and Fe^{3+} (ferric, oxidized form of Fe), and (b) its ability to form octahedral complexes with various ligands and to vary its redox potential in response to different environments. Fe availability is indeed the function of solubility rather than of its abundance in the soil (Sahrawat 2000; Guerinot 2001; Hell and Stephan 2003; Pirzadah et al. 2010). Zinc deficiency is common in soils with neutral and alkaline pH (calcareous, saline-sodic, and sodic soils), intensively cropped soils, soils with poor drainage, and lowland rice soils (Marschner 1995; Fageria et al. 2002; Alloway 2009; Koegel-Knabner et al. 2010; Pirzadah et al. 2010).

The ability of plants to translocate Fe and Zn in seed is controlled by a homeostatic mechanism in the plant that regulates absorption, translocation, and phloem sap loading-unloading rates of Fe and Zn (Marschner 1995; Welch 1995; Mori 1999; Schurr 1999; Hell and Stephan 2003; Borg et al. 2009). Iron homeostasis is especially well controlled due to the redox potential of free iron radicals and due to the high concentration of iron in most acid tropical soils where many of the world's crops originated (maize, common bean, sorghum, chickpea). Zinc, on the other hand, is more often taken up without strict control mechanism due to its generalized deficiency in many tropical and temperate soils, especially those of volcanic or loess origin.

An interaction of micronutrients with macronutrients needed by crops is also of importance. Hao et al. (2007) conducted a pot experiment to study the effects of nitrogen (N) fertilization on the distribution of Fe and Zn in rice shoot and seed of two rice cultivars, IR68114 and

IR64. IR68114 is a sister line of IR64 bred for high seed-Fe density. In comparison to the control (0 N applied), the application of N fertilizer (80, 160, and 320 kg N ha^{-1}) increased the concentrations of minerals including Fe and Zn in plant parts as a result of their improved transport from roots to shoots with N application. The two cultivars differed in the accumulation and concentration of micronutrients indicating that the characteristic expression of the two rice genotypes was not controlled by the amount of N fertilizer added. The concentrations of Fe and Zn in brown rice due to N application increased by 29% and 16% for IR64 and by 22% and 20% for IR68144. The results of this study suggest that the application of nutrients other than Fe and Zn–especially N that is generally universally deficient in soils–is equally important for improving the seed Fe and Zn in cereals such as rice. More recently, similar results on the effects of N application in improving the seed-Fe and -Zn concentrations were also reported for durum wheat (Cakmak et al. 2010; Kutman et al. 2010). These authors report that N nutritional status of the wheat plants can have a synergistic impact on root uptake and the deposition of Fe and Zn in seed when N, Zn, and Fe are applied together (Alloway 2009). Similar studies with phosphorus (P) and potassium (K) supply are under way in common bean (Blair et al. 2009a). It is known that in situations with multiple nutrient deficiencies, balanced plant nutrition (combined application of all nutrients that are deficient in the soil) is a prerequisite for improving productivity and nutritional quality of seeds of cereals (Rego et al. 2007; Sahrawat et al. 2008; Pirzadah et al. 2010).

Genotypic differences also exist in the allocation of micronutrients such as Fe and Zn to seed and nonseed parts. For example, Wu et al. (2010) studied the uptake, translocation, and remobilization of Zn absorbed at different growth stages by rice genotypes of different Zn densities using Zn^{68} stable isotope tracer. They found that significant differences in Zn allocation existed between two rice genotypes. Higher Zn concentrations were found in seeds, stems, and leaves of cultivar IR68144 than in IR64, but higher Zn was found in roots of IR64. More than half of the Zn accumulated in the seeds was remobilized before anthesis, and accounted for 63% and 52% of the total Zn uptake for IR68144 and IR64, respectively. The results of this study indicate that Zn density in rice seeds is closely associated with the ability to translocation of Zn from old tissues to new tissues at both early and late growth stages of the rice crop and with phloem remobilization of Zn from nonseeds parts, especially leaves and stems to seeds (Wu et al. 2010). These results are in agreement with those reported by Haslett et al. (2001) who found that foliar application of Zn (applied as

Zn^{65} labeled isotope tracer) in inorganic and organic form is equally suitable for providing adequate Zn nutrition to the wheat plant, thus demonstrating the phloem transport of Zn from leaves to roots of the wheat plants.

The results of the study by Haslett et al. (2001) along with those reported by Pearson and Rengel (1994, 1995) clearly demonstrate that the transport of Zn in phloem from the stem and lower leaves and roots is significant. The results also establish that phloem transport of Zn from leaves and stem to the developing seed is an effective mechanism for the accumulation of Zn in wheat seeds. It was concluded that Zn is highly mobile in phloem (Pearson and Rengel 1994, 1995; Haslett et al. 2001). Similarly, the transport of iron in phloem is well studied although the transport mechanism is not well known and may involve citric acid conjugates and/or nicotianamine as a transport system with most of the studies on Fe-transport based on mutants in peas (Grusak 2000, 2002) or *Arabidopsis* (Grotz and Guerinot 2006), or rice (Takahashi et al. 2001; Cheng et al. 2007; Masuda et al. 2009). Iron-uptake through iron reductase activity (IRA) seems to be important for total seed accumulation of this mineral in common bean (Blair et al. 2010a).

It has also been reported that the amounts of Zn uptake vary among cereals such as rice, wheat, and barley. In some species such as rice, continuous Zn uptake during seed filling and continuity of loading into the endosperm from the xylem might be the key process. Also, continued Zn uptake requires genetically improved uptake capacity along with Zn availability in the soil or the growing medium (Zee and O'Brian 1970; Pearson and Rengel 1995; Krishnan and Dayanandan 2003; Alloway 2009).

In species such as rice, the root-to-shoot transfer appears less important than seed loading. It would seem for rice at least that endosperm loading might be low, not so much because of transport barriers but because of limited uptake or sink capacity in the highly starch-filled cells (Stomph et al. 2009). Hence for rice, the sink capacity needs to be enhanced by increasing the nonstarch to starch ratio in the endosperm through a larger number of cells with slightly reduced size while maintaining seed production (Stomph et al. 2009). Whether the same is true in the case of wheat and barley is an important issue for future study, and obviously there is a need to generate results on the comparative evaluation of these crops with rice (Stomph et al. 2009).

In wheat plants, remobilization from leaves is important for Zn allocation to seed and Zn is phloem mobile (Marschner 1995; Pearson and Rengel 1995). On the other hand, in rice, xylem-transported Zn from uptake during seed filling might be more important

for Zn accumulation in seed than phloem-transported Zn remobilized from leaves (Ziang et al. 2007, 2008). It would seem that contrary to wheat (Zee and O'Brian 1970), there is no xylem discontinuity in the rice plant (Zee 1972; Krishnan and Dayanandan 2003). Since rice plant xylem is continuous, Zn can be loaded directly from the xylem in the vascular bundle to the nucellar epidermis and aleurone cells. In dicotyledonous legumes where the pod wall is continuous with stem xylem but the seed embryo is not this may vary. Iron uptake in the meantime is well understood but its transport through phloem and xylem less so. This is especially important in the case of seed loading of iron, where the mechanism is almost completely unknown but must be controlled by a mixture of sink strength and supply. Remobilization from leaves to stem and then to seeds involves the breakdown and creation of different ferritins expressed in each tissue.

It is clear from this discussion that research on the uptake, translocation, and deposition of minerals such as Fe and Zn in seed has focused mainly on wheat, rice, and to some extent, maize to understand the mechanisms involved in the biofortification of crops. There is hardly any information, although needed for understanding the process of biofortification, on these aspects for other important food crops, including pearl millet and sorghum in the semiarid tropical regions of Asia and Africa. Obviously, there is a need to investigate the process of seed loading for other cereals as the information would be useful to ascertain differences caused by the differences in the anatomy (Thorne 1985).

In addition to the physiological mechanisms involved in the uptake, distribution, and accumulation of Fe and Zn in seed (Borg et al. 2009; Cakmak et al. 2010), the use of genes (Lönnerdal 2003; Vasconcelos et al. 2003; Ghandilyan et al. 2006; Brinch-Pedersen et al. 2007; Waters et al. 2009; Sperotto et al. 2010), and chelating agents such as EDTA (Nowack et al. 2008) have been reported to enhance efflux of nutrients including Fe and Zn from the vegetative tissues to seed of crops.

E. Nutrient Use Efficiency

Micronutrient availability in the soil, especially of Fe and Zn, is influenced by a range of soil, plant, and climatic factors (Fageria et al. 2002). The Fe and Zn deficiency-related problems in various soil orders are further influenced by the nature of the plant material from which the soil is developed (Dudal 1976). Nutrient-use efficiency consists of two components, one related to crop productivity and the second that emphasizes the internal nutrient requirements for a crop or

genotype. Relative to productivity, nutrient-use efficiency may be defined as the ability to produce higher yield in a soil that otherwise would limit the productivity of a standard or control genotype (Marschner 1995). In terms of agronomic efficiency, it can simply be defined as yield per unit of nutrient (Fe and Zn in this case). Relative to internal requirement or the physiological efficiency for a nutrient, it can be defined as the yield (economic or total) per unit uptake of the nutrient (for detailed discussion, see Gourley et al. 1994; Marschner 1995; Baligar et al. 2001; Rengel 2001; Fageria et al. 2008; Khoshgoftarmanesh et al. 2010). For the purpose of this section for selecting nutrient (Fe and Zn) rich seeds, we interpret the Fe and Zn efficiencies of a crop or genotype in terms of seed concentrations of these nutrients. Harvest index (HI) as defined as a component of seed yield is the ratio of seed mass by total biomass, while Fe or Zn HI is the ratio of Fe or Zn concentration in the seed divided by the total concentration of Fe or Zn in the biomass. It is thus the Fe or Zn HI that matter when selecting for seed mineral-dense crops: higher HI for Fe and Zn more of these nutrients in seeds. To breed seed mineral-dense crops with high productivity, selection should be based on combining high $HI_{seed\ yield}$ together with high $HI_{seed\ Fe\ or\ Zn}$.

In practical terms when grown on a soil with low availability of Fe or Zn, a micronutrient-efficient genotype acquires and uses a higher amount of these minerals for seed yield as compared to the inefficient genotype, which can be termed nutrient acquisition and use efficiency, respectively. These genotypic differences can arise from greater uptake or greater allocation of the micronutrients to the seed where they allow for better seedling establishment (Baligar et al. 2001; Fageria et al. 2008; Khoshgoftarmanesh et al. 2010). In calcareous soils, rye, triticale, bread, and durum wheat showed differential response to Zn efficiency, rye being exceptional in its high Zn efficiency, followed by triticale > bread wheat > durum wheat, which could be attributed to its greater Zn uptake capacity from soils (Cakmak et al. 1997b; Erenoglu et al. 1999). The exceptionally high Zn efficiency of rye is because several of its chromosomes, particularly 1R and 7R, carry the genes controlling Zn efficiency, which are transferable into wheat and can be used for development of new wheat cultivars with high Zn efficiency for Zn-deficient soils (Cakmak et al. 1997a). Further, domesticated emmer wheat accessions such as 3717, 19385, and 22287 were reported to be more Zn efficient (g dry matter/Zn concentration) than modern durum and bread wheats (Genc and McDonald 2008).

Fageria and Baligar (2005) conducted a greenhouse study to evaluate Zn-use efficiency of 10 upland rice genotypes on an Oxisol under

two Zn treatments: no Zn and application of 10 mg Zn kg^{-1} soil as zinc sulfate. The results revealed that shoot dry weight, seed yield, HI_{Zn}, Zn concentration in shoot and in seed were significantly influenced by soil Zn level and varied by genotype. However, $HI_{seed\ yield}$ was significantly affected only by genotype. Genotypes also differed significantly in Zn recovery efficiency and on average 13% of the applied Zn was recovered by upland rice genotypes. Seed-Zn concentration varied from 26 to 41 mg kg^{-1}. $HI_{seed\ yield}$ varied from 0.38 to 0.53 with an average value of 0.46. The HI_{Zn} varied from 0.40 to 0.76 with an average value of 0.60 under no application of Zn fertilizer; HI_{Zn} decreased with applied Zn, and varied from 0.21 to 0.37 with a mean value of 0.28. The results show that upland rice genotypes differ significantly in seed yield, seed Zn and HI_{Zn} both with and without the application of Zn.

In experiments with rice conducted under controlled conditions, Jiang et al. (2008) reported that with increasing Zn supply, the Zn concentration in all individual plant organs increased, but the increase in Zn concentration in stems and rachis was much larger than in seeds. Over a range of added Zn, the Zn concentration in stems increased from 20 to 400 mg kg^{-1}, but the concentration in the brown rice increased only from 20 to 50 mg kg^{-1}. The HI_{Zn} in the tested rice cultivars decreased considerably with increasing total plant Zn content probably due to concentration effect (opposite of dilution). These results are consistent with those reported earlier for wheat (Herren and Feller 1994) and rice (Fageria and Baligar 2005). Furthermore, Jiang et al. (2008) demonstrated that due to physiological regulation and barriers in the rice plant, it is difficult to enhance Zn concentration in brown rice by simply increasing Zn supply in the soil or growing medium. It has been proposed that while the breeding target could be to enhance the level of maximum Zn accumulation in the overall plant, further research should focus into the exact tissues in which the regulation of Zn translocation to seed is strongest and into the genes involved in the regulation mechanism for zinc transport (Jiang et al. 2008).

Sahrawat (2000) determined the amounts of macro- and micronutrients removed by cultivars in a field experiment conducted with an upland rice cultivar, WAB 56-50, under rainfed upland or a lowland rice cultivar, Bouake 189, under irrigated lowland conditions in Ivory Coast, West Africa. The nutrient HI was highest for P (0.69) and lowest for K (0.10). Both HI_{Fe} and HI_{Zn} were greater for the lowland rice (HI_{Fe} 0.46, HI_{Zn} 0.50) as compared to the upland rice cultivars (HI_{Fe} 0.21, HI_{Zn} 0.38). Clearly, the lowland rice ecology provides a better growing soil

environment than does the upland ecology for rice growth and yield and Fe or Zn accumulation by the crop. Furthermore, it has been demonstrated that the availability of nutrients especially of Fe and Zn to rice in soils is greatly influenced by water regime (including flooding) and the availability of Fe and Zn is generally favorably affected in the lowland irrigated agroecologies (Ponnamperuma 1972; Gao et al. 2006; Sahrawat 2007, 2009).

In summary, standardized methodology for rapid and effective screens of germplasm pool is a prerequisite for identifying and developing crop cultivars dense in seed Fe and Zn under well-defined or optimum nutrient conditions, especially nutrients other than Fe and Zn. The various screening methods used vary from those conducted under controlled conditions to real world practical conditions in the field, using soil as the substrate. Ideally, the amounts of other nutrients (other than Fe and Zn) including major, secondary, and micronutrients are kept in the optimum, while the concentrations of Fe or Zn cover the entire deficiency to the sufficiency range. For practical breeding purposes, identification of plant traits associated with increased acquisition of Fe and Zn from the growing medium, generally soil, is of critical importance. The selection of traits varies with soil type, especially with soil pH (acidic to alkaline soil reaction) and soil water status. Despite diverse soil and agroclimatic conditions under which crops or their cultivars are screened for grains denser in Fe and Zn, it has been established that genotypes indeed vary in Fe and Zn uptake, accumulation, and translocation to seed and nonseed parts of crops. To date, the research on uptake, translocation, deposition, and use efficiency of minerals such as Fe and Zn has been mostly confined to wheat, rice, and to a lesser extent maize. Little information, although urgently needed to understand the process of biofortification, is available for other important food crops such as sorghum and pearl millet. Future in-depth basic research need to focus particularly on understanding the process of seed loading especially in cereals other than wheat and rice, as such insights would help to ascertain differences in seed Fe and Zn associated with the differences in the anatomy. Rapid, simple, and cost-effective methods for routine determination of β-carotene, Fe and Zn are needed so that a large number of germplasm/breeding lines can be screened and information generated in a timely manner. XRF is an effective assay for initial screening of seed Fe and Zn to discard lines in the lower range, while semiquantitative analysis instead of HPLC may be used to discriminate lines for variation in β-carotene; however, promising lines from these initial screens must be analyzed by ICP or AAS assays for Fe/Zn or by HPLC for β-carotene.

V. MINING GERMPLASM COLLECTIONS FOR NATURAL VARIATION FOR SEED IRON, ZINC, AND PHYTATE

A. Variation and/or Bioavailability of Seed Iron, Zinc, and Phytate

1. Seed Iron and Zinc Concentration. Natural variation in plant genetic resources provides the basic raw material and plays a fundamental role in crop improvement programs. Published evidence on screening of germplasm revealed substantial variation in seed-Fe and -Zn concentrations in common bean, maize, pearl millet, rice, sorghum, and wheat (Table 3.1). For example, higher Fe in some accessions of common bean germplasm from Colombia, Chile, Peru, Rwanda, and Tanzania has been identified; high Fe and Zn was found in maize germplasm from southern Africa; high Fe and Zn in pearl millet was identified in *Iniadi* landraces from West Africa; high Fe reported in farmer's preferred sorghum varieties from Benin; traditional rice cultivars were found to contain more Fe and Zn than modern cultivars; and high seed-Fe and -Zn was found in einkorn wheat, wild emmer wheat, and species with S and D genomes. Gene banks in CGIAR centers hold large collections of both cultivated and wild relatives of their mandate crops, with CIAT, CIMMYT, ICARDA, ICRISAT, IRRI, and Africa Rice Center together maintain a total of 7,41,319 accessions of 3,446 species of 612 different genera (http://singer.cgiar.org/). Only a fraction of the germplasm preserved in gene banks has been screened for seed-Fe and -Zn concentrations (or contents). Clearly, there is a greater need to assess for natural genetic variability locked in these germplasm collections. However, in most cases, it will not be possible for any institution to screen the entire germplasm collection of a given species because of enormous cost and technical manpower associated with the analysis of seed samples for chemical characteristics. Forming core and/or mini-core collections is one way to sample the representative variability from the entire collection of a given species, thus providing an entry point for a wider search in the entire collection (Brown 1989; Upadhyaya and Ortiz 2001). Such subsets have been reported for most major cereal and legume crops (reviewed in Dwivedi et al. 2005, 2007), and should be evaluated to assess the range of genetic variation in seed Fe and Zn for use in crop breeding. Islam et al. (2002, 2004) analyzed a partial core collection of common bean for variability in Fe and Zn traits while Blair et al. (2010b) did the same for a Rwandan collection that was conserved prior to genocide in that country. Astudillo and Blair (2008), meanwhile, evaluated the Fe and Zn concentrations of all released bush bean varieties in Colombia as a first approximation of micronutrient variability in local germplasm. Likewise,

Table 3.1. Natural genetic variation for seed-Fe and -Zn concentrations in common bean, groundnut, maize, pearl millet, rice, sorghum, and wheat germplasm and cultivars covering a period from 1997 to 2010.

Germplasm evaluated	Summary of the variation in seed mineral content	Range variation	References
Common bean			
29 U.S. grown cultivars and CIAT breeding lines	Fe 8.9–112.9 and Zn 30.9–64.6 mg kg^{-1}	Fe 104 and Zn 33.7 mg kg^{-1}	Akond et al. 2011
365 Rwandan landraces	Fe 45.3–95.6 and Zn 25.1–49.1 ppm	Fe 50.3 and Zn 24.0 ppm	Blair et al. 2010c
29 U.S. cultivars/high Fe lines	Fe 30.9–64.6 and Zn 8.9–112.9 ppm	Fe 33.7 and Zn 104 ppm	Talukder et al. 2010
155 Lines from Portugal	Fe 32.2–88.4 and Zn 11.5–45.3 ppm	Fe 56.2 and Zn 33.8 ppm	Pinheiro et al. 2010
90 Lines from Tanzania	Fe 23.6–105.5 and Zn 19.0–56.1 ppm	Fe 81.9 and Zn 37.1 ppm	Tryphone and Nchimbi-Msolla 2010
55 Lines from Chile	Fe 68.9–152.4 and Zn 27.9–40.7 mg kg^{-1}	Fe 83.5 and Zn 12.8 ppm	Paredes et al. 2009
>1,000 Germplasm (mostly CIAT core)	Fe 34–89 and Zn 21–54 μg g^{-1}; Peru germplasm exceptionally high iron (averaged >100 μg g^{-1})	Fe 55 and Zn 33 μg g^{-1}	Graham et al. 1999
Groundnut			
9 Diverse lines	Fe 13.4–17.9 and Zn 25.2–29.8 μg g^{-1}	Fe 4.5 and Zn 4.6 μg g^{-1}	Phan-Thien et al. 2010
Maize			
>1,800 Germplasm	Landraces: Fe 17.5–58.5 and Zn 14.9–29.7 mg kg^{-1} Southern Africa: Fe 16.4–63.2 and Zn 12.9–57.6 mg kg^{-1}	Landraces: Fe 41 and Zn 14.8 mg kg^{-1} Southern Africa: Fe 46.8 and Zn 44.7 mg kg^{-1}	Bänziger and Long 2000

Pearl millet			
90 Lines	Fe 30.1–75.7 and Zn 24.5–64.8 mg kg^{-1}, *Iniari* landraces high in Fe	Fe 45.6 and Zn 40.3 mg kg^{-1}	Velu et al. 2007
10 Accessions	Fe 70–180 and Zn 53–70 $\mu g\, g^{-1}$	Fe 110 and Zn 17 $\mu g\, g^{-1}$	Abdalla et al. 1998
Rice			
202 Modern/traditional cultivars	Traditional cultivars: Fe 1.2–39.2 and Zn 3.0–38.6 mg kg^{-1}; modern cultivars: Fe 4.1–20.6 and Zn 3.4–25.8 mg kg^{-1}	Traditional cultivars: Fe 38.9 and Zn 35.6 mg kg^{-1}; modern cultivars: Fe 16.5 and Zn 22.4 mg kg^{-1}	Anandan et al. 2011
25 Germplasm	Fe 17–361 and Zn 30–64 ppm	Fe 344 and Zn 30 ppm	Singh et al. 2010b
35 Upland cultivars	Fe 14.5–31.4 and Zn 24.4–45.1 mg kg^{-1}	Fe 16.9 and Zn 20.7 mg kg^{-1}	Moraes et al. 2010
Wild *Oryza*	Fe in brown rice 14–47 and 5–25 $\mu g\, g^{-1}$ in milled rice; Zn in brown rice 35–56 and 28–46 $\mu g\, g^{-1}$ in milled rice; more Fe and Zn in wild than cultivated spp.	Brown rice Fe 33 and 20 $\mu g\, g^{-1}$ in milled rice; brown rice Zn 21 and 18 $\mu g\, g^{-1}$ in milled rice	Jiang et al. 2009
12 Diverse lines including wild relatives	Fe 8.5–20.5 and Zn 13.9–39.3 $\mu g\, g^{-1}$	Fe 12.0 and Zn 25.3 $\mu g\, g^{-1}$	Chandel et al. 2010
40 Commercial varieties	Fe 8.8–16.3 and Zn 19–36 ppm	Fe 7.8 and Zn 17 ppm	http://webapp.ciat.cgiar/epmr_ciat/pdf/poster_19_epmr07pdf
Traditional varieties	Fe 9–21 and Zn 14–36 ppm	Fe 12 and Zn 12 ppm	Graham et al. 1999; Gregorio et al. 2000
1,138 Germplasm	Fe 6.3–24.4 and Zn 13.5–58.4 $\mu g\, g^{-1}$; more Fe in aromatic rice/traditional cultivar, Jalmagna	Fe 18.1 and Zn 44.9 $\mu g\, g^{-1}$	

(*continued*)

Table 3.1 (*Continued*)

Germplasm evaluated	Summary of the variation in seed mineral content	Range variation	References
Sorghum			
20 Commercial hybrids	Fe 30–44 and Zn 22–33 ppm	Fe 14 and Zn 11 ppm	Kumar et al. 2010a
29 Lines from core collection	Fe 26–61 and Zn 21–57 mg kg^{-1} seeds	Fe 35 and Zn 36 mg kg^{-1} seeds	Kumar et al. 2009
76 Farmers varieties from Benin	Fe 30–113 and Zn 11–44 mg kg^{-1} seeds	Fe 83 and Zn 33 mg kg^{-1} seeds	Kayodé et al. 2006
84 Diverse lines	Fe 20–37 and Zn 13.4–31.0 ppm	Fe 17 and 17.6 ppm	Reddy et al. 2005
Wheat			
600 Core collection accessions	Fe 26–69 and Zn 17–61 mg kg^{-1} seeds	Fe 43 and Zn 44 mg kg^{-1} seeds	Velu et al. 2011
154 Cultivars/wild emmer	Fe 12–112 and Zn 22–70 mg kg^{-1}; two fold greater variation in wild emmer than wheat cultivars	Fe 100 and Zn 48 mg kg^{-1}	Chatzav et al. 2010
19 Wild emmer	Fe 27–86 and Zn 39–112 mg kg^{-1} seeds	Fe 59 and Zn 73 mg kg^{-1}	Gómez-Becerra et al. 2010
84 Durum wheat cultivars	Fe 33.6–65.6 and Zn 28.5–46.3 mg kg^{-1} seeds	Fe 32 and Zn 17.8 mg kg^{-1}	Ficco et al. 2009
150 Bread wheat lines	Fe 28.9–50.8 and Zn 13.5–34.5 mg kg^{-1}	Fe 31.9 and Zn 31 mg kg^{-1}	Zhao et al. 2009
265 Germplasm from China	Fe 28–65 and Zn 21–58 mg kg^{-1} seed	Fe 37 and Zn 37 mg kg^{-1}	Zhang et al. 2010
66 Spring and winter cultivars	Fe 25–56 and Zn 20–39 mg kg^{-1} seed; spring wheat more Fe while winter wheat more Zn	Fe 31 and Zn 19 mg kg^{-1}	Morgounov et al. 2007
D, US and ABD genome accessions	More Fe (51–109 ppm) and Zn (37–115 ppm) in *A. kotschyi*, *A. tauschii* and synthetics than cultivated wheat (Fe 30–44 ppm and Zn 21–30 ppm)	Fe 58 and Zn 78 ppm	Chhuneja et al. 2006
825 Einkorn accessions	Fe 15–109 and Zn 14–190 mg kg^{-1}	Fe 94 and Zn 176 mg kg^{-1}	Cakmak et al. 2004

the seed-Fe and -Zn concentrations among selected sorghum core collection accessions varied, respectively, from 26 to 60 and 21 to 57 mg kg^{-1} seeds compared to controls (Fe 40 mg kg^{-1} and Zn 24 mg kg^{-1} seeds) (Kumar et al. 2009).

Another approach would be to explore the genetic variation of mineral concentration in germplasm from geographic regions deficient in soil micronutrients, given that germplasm from such regions is expected to develop inherent adaptation mechanisms that favor enhanced nutrient uptake, transport, distribution, and relocation in plants/seeds. This approach, which is also known as habitat characterization or focused identification of germplasm selection (FIGS) (Street et al. 2008), has been successfully employed to characterize plant habitats and species' adaptive responses to temperature, day length, and stresses (Berger 2007; Kaur et al. 2008; Bhullar et al. 2009; El Bouhssini et al. 2009; Berger et al. 2011).

Wild and weedy relatives of common bean and wheat have shown large variability for seed-Fe and -Zn concentrations (Cakmak et al. 2000; Guzmán-Maldonado et al. 2000; Chhuneja et al. 2006; Acosta-Gallegos et al. 2007; Xie and Nevo 2008). For example, the 75 wild and weedy common beans from Jalisco and Durango state of Mexico showed large variability for seed Ca (500–7470 mg kg^{-1}), iron (64–280 mg kg^{-1}), and zinc (11–33 mg kg^{-1}) concentrations (Guzmán-Maldonado et al. 2000) or nonprogenitor *Aegilop* species of wheat with large variability for grain Fe and Zn (Chhuneja et al. 2006).

Cheng et al. (2007) isolated a point mutation in a gene encoding nicotianamine aminotransferase (NAAT1), which disrupted strategy II system of rice due to the loss of a functional NAAT enzyme, and the *naat1* mutant exhibited 3.8-fold higher Fe than the WT. More importantly, the Fe concentration of the polished seed of the *naat1* mutant from field grown rice plants was 4.6 mg kg^{-1}, that is, 3.8-fold higher than the wild type approaching that of the highest naturally existing lines (Barry 2006). This rice mutant–along with other natural occurring high seed-Fe concentration germplasm–is an ideal resource to enhance seed Fe into the improved genetic background.

2. Seed Phytate Concentration. Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate or InsP6) is the major form of phosphorus storage, which accounts for 65%–85% of the total phosphorus in mature seeds (Raboy 1997). High concentration of phytic acid in foods limits micronutrient bioavailability as it binds minerals (Ca, Fe, and Zn) to form mixed salts (phytin), largely excreted by humans and nonruminant animals. The excreted phytin significantly impacts water pollution

(Lott et al. 2000; Raboy 2001). However, phytic acid is vital for seed development, seedling growth, and development and may have a positive role as antioxidant and anticancer agent (Oatway et al. 2001). The development of lp seed crops is one of several ways to improve the nutritional quality as well as developing environment-friendly, sustainable production systems. Low phytic acid crops may offer improved nutrition for human populations that largely depend upon cereal- and legume-based staple foods.

Efforts have been made to reduce the levels of phytate in seeds by exploiting natural genetic variation as well by induced mutations. Studies of germplasm collections have revealed significant genetic variation for both seed phytate and/or total P in common bean, lentil, sorghum, soybean, and wheat (Lolas and Markakis 1975; Raboy et al. 1991; Israel et al. 2006; Kayodé et al. 2006; Ficco et al. 2009; Thavarajah et al. 2010; Akond et al. 2011). Likewise, researchers have used both chemical and physical mutagens to isolate lp mutants in barley (Raboy 2000; Bowen et al. 2006), common bean (Campion et al. 2009), maize (Raboy 2000; Badone et al. 2010), rice (Larson et al. 2000; Raboy 2000; Rutger et al. 2004; Liu et al. 2007; Kim et al. 2008), soybean (Wilcox et al. 2000; Walker et al. 2006), and wheat (Guttieri et al. 2004). These studies reported two types of mutants, those with moderate reduction (50%–65%) or extreme reduction (95%) in phytic acid, with the latter being homozygotes and lethal. The total seed phosphorus in these mutants was unaffected, while reduction in phytic acid resulted in corresponding increases in inorganic P in the seed. However, lp mutants, in general, had reduced germination and seedling development and yielded low compared to WT (Meis et al. 2003; Pilu et al. 2003; Oltmans et al. 2005; Shi et al. 2007), with some exception in barley (Bregitzer and Raboy 2006), soybean (Yuan et al. 2007), and common bean (Campion et al. 2009). Badone et al. (2010) isolated a low phytic acid mutant in maize, lp1–241, with a reduction of up to 90% of phytic acid and strong pleiotropic effect on the whole plant including higher level of anthocyanins as compared to wild type either in the embryo (~3.8-fold) or in the aleurone layer (~0.3-fold). These mutants could help to discover the carriers and the regulatory mechanisms involved in the vascular transport of plant cell and xenobiotic molecules involved in several fundamental processes, which so far are not fully understood. More recently, Akond et al. (2011) reported large variation in phytic acid (12.52–316.42 mg kg^{-1}) among the 29 U.S. grown cultivars and CIAT breeding lines in common bean, with low phytic acid genotypes such as JaloEEP558, Vista, Xan176, Albion, Voyger, and G122 having high levels of minerals (Ca, Fe, Zn) concentration. Large germplasm collections of the major food crops are preserved in gene banks

globally, but only a fraction of these collections have been screened for low seed phytate. It is reasonable to believe to find genetic variants in these germplasm collections that have low phytate and acceptable agronomic performance. Screening reduced subsets, such as core or mini-core subsets, could be seen as a starting point to mine variation for low seed phytate in germplasm collections.

3. Seed Iron and Zinc Bioavailability. Phytic acid is the major contributor to reduced bioavailability of Fe and Zn in cereals and legumes. Few germplasm lines with exceptionally high seed-Fe and/or -Zn concentrations have been reported in common bean, maize, pearl millet, rice, sorghum, and wheat (Table 3.1). However, very little is known about how much of the seed Fe or Zn is bioavailable for absorption. Using a range of common bean genotypes differing in seed-Fe concentrations and rat model for bioaccessibility, Welch et al. (2000a) detected large differences in Fe bioavailability, ranging from 53% to 76% of total Fe, with higher seed-Fe genotypes resulting in increased amounts of total bioavailable Fe. Likewise, Zn bioavailability in wheat genotypes ranged from 60% to 82% (Welch et al. 2000b). Significant differences in seed Fe and/or Zn bioavailability were also reported for maize germplasm (Oikeh et al. 2003a,b, 2004a; Šimic et al. 2009). For example, Oikeh et al. (2003a) detected large variation in Fe bioavailability that ranged from 30% below to 88% above the reference control cultivar, TZB-SR. In some maize cultivars with high seed-Fe and -Zn concentrations (22–24 mg kg^{-1}) the bioavailable Fe was 24%–36% higher than the reference control (Oikeh et al. 2004a). Wheat *Aegilops* species and their derivatives are reported to possess high grain Fe and Zn concentrations (Chhuneja et al. 2006; Neelam et al. 2010a; Tiwari et al. 2010). More recently, Salunke et al. (2011) detected larger bioavailable Fe among wheat *Aegilops* derivatives selected for high grain Fe and protein concentrations. The bioavailable Fe among these derivatives increased up to 1.5-fold, corresponding to a 1.5- to 2.2-fold increase observed in grain Fe over control. Clearly, more studies are needed to identify genotypic variation for seed Fe and Zn bioavailability in order to select genotypes, which have not only high seed-Fe and -Zn concentrations but also more bioavailable Fe/Zn for absorption.

B. Distribution of Iron and Zinc in the Seed

Understanding the accumulation and distribution of essential nutrients in the seed is of primary importance for improving the nutritional quality of staple crops. Information about the micronutrient

distribution can be obtained using *in situ* staining or spectroscopic techniques. Staining methods target specific metals, based on chemical reactions between the histological dye and the metal of interest. However, these reactions are subject to competitive exchange equilibrium with endogenous ligands and are usually considered liable to visualize only labile ions (McRae et al. 2009). Spectroscopic methods include proton-induced X-ray emission (PIXE) that targets the embryo region (Mazzolini et al. 1985), scanning and transmission electron microscopy (STEM) in combination with energy dispersive X-ray microanalysis (EDX) that focuses on aleurone and scutellum cells to provide subcellular information (Ockenden et al. 2004; Lombi et al. 2010), nano-secondary ion mass spectrometry (nano-SIMS) that visualizes the subcellular distribution but is limited to regions of only a few μm^2 (Moore et al. 2010), and the X-ray fluorescence (XRF) method that provides elemental maps for various elements in whole grain sections (Lombi et al. 2009; Takahashi et al. 2009). The nondestructive μ-XRF technique permits a three-dimensional reconstruction of accumulation patterns and can also distinguish between ionic valencies, critical for accumulation of toxic forms of various ions (Scheckel et al. 2007). More recently, Ryan et al. (2010) developed a large energy-dispersive detector array called, Maia, to capture intricate detail in natural material, together with faster acquisition and improved counting statistics in elemental imaging. A 96-detector prototype demonstrated the capacity of the system for real-time deconvolution of complex spectral data using an embedded implementation of the dynamic analysis method that acquires highly detailed images of up to 77 M pixels spanning large areas of complex material sample sections. An excellent review by Lombi et al. (2010) focuses on the most recent status of *in situ* techniques to visualize spatial distributions and assess the speciation of metals and metalloids. Sample preparation probably constitutes the most critical step and is method (and to some extent also species-) dependent. The above-mentioned techniques differ in terms of resolution and sensitivity, depth of analysis, and in their capacity to provide mass resolution or molecular information.

Detailed knowledge of the distribution of macro- and micronutrients provides indications on possible ligands controlling the bioavailability of certain elements, such as Fe and Zn, and this information may also be useful to minimize the losses during milling/polishing. The wheat seed at maturity consists of an outer layer of maternal origin comprising a testa derived from the integuments, the pericarp, and awns, while the central tissues consist of an embryo and endosperm, respectively, derived from single and double fertilization events. The outermost

layer of the endosperm is differentiated into an aleurone. The endosperm is filled with starch and storage proteins, while the aleurone and the embryo accumulate a range of nutritional reserves such as minerals, carbohydrates, fats, and proteins, including enzymes. Mazzolini et al. (1985) found high concentrations of iron in wheat seed aleurone and the scutellum layer of the embryo and low concentrations in the endosperm. Barley endosperm and aleurone in contrast, together contain ~70% of the total Fe, but only 7%–8% in the embryo (Duffus and Rosie 1976). Zinc in wheat is predominantly located in the embryo and aleurone parts of the seed (Ozturk et al. 2006). The Zn concentration in seeds is particularly high during early seed development (i.e., at milking stage); thereafter, its concentration gradually declines until maturity. Using ear culture system (Singh and Jenner 1983; Sharma et al. 1995), the stable zinc isotope ^{70}Zn, and laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) technique, Wang et al. (2011) detected preferential localization of Zn in the aleurone and embryo, as also observed by Ozturk et al. (2006), but found a gradient of ^{70}Zn concentration between crease vascular tissue, aleurone layer, and endosperm, demonstrating that Zn is distributed within the seed through the crease phloem. This suggests that two barriers of Zn transport into wheat seeds may exist: between the stem tissue rachis and the seed, and between the maternal and filial tissues in the seed. Likewise, large gradients in the distribution of micronutrients were reported for both within and between different tissues of barley seed, with gradients especially evident in the embryo and the scutellum regions (Lombi et al. 2011). Moreover, the ventral and dorsal part of the barley seeds also showed significant differences in element distribution. Furthermore, the speciation analysis of barley seed tissues using SEC-ICP-MS and IP-ICP-MS techniques revealed highest concentrations of Zn, Fe, S, and P in the bran and embryo fractions (Persson et al. 2009). Analysis of the embryo further revealed differences in speciation of Fe and Zn. The majority of the Fe coeluted with P as a species with the apparent mass of 12.3 kDa, whereas the majority of Zn coeluted with S as a 3 kDa species, devoid of any coeluting with P. These results show that Zn appears to be bound mainly to peptides, whereas Fe is associated mainly with phytic acid.

Element-specific distribution patterns of micronutrients have been reported for rice seeds. Lombi et al. (2009) detected high concentrations of Cu, Fe, Mn, and Zn in certain regions of the husk. However, the distribution of these nutrients varied considerably in other parts of the seed. For example, Zn in the central part of the embryo, which likely corresponds to the plumule; however, its concentration deceases

gradually from the aleurone/pericarp and outer parts of the endosperm to the interior of the endosperm, while Mn and Fe very much localize in the aleurone/pericarp region with a sharp change in the concentration in the exterior parts of the endosperm. Mn is highly concentrated in the embryo but with a different pattern than observed for Zn. The strong similarities between the distribution of Fe, Mn, and P and between Zn and S may be indicative of the complexation mechanisms involved in rice seeds. Preliminary studies in pearl millet revealed greater concentration of seed minerals including Fe and Zn in the covering layers and the germ than in the endosperm portions, similar to most cereal seeds (Varriano-Marston and Hoseney 1980).

Common bean and soybean genotypes were reported to accumulate different proportion of total seed Fe in the seed coat, embryo, and cotyledons (Laszlo 1990; Moraghan and Grafton 2002; Moraghan et al. 2002; Moraghan 2004; Ariza-Nieto et al. 2007; Cvitanich et al. 2010, 2011), indicating that specific tissues relevant for Fe storage should be identified and their Fe loading mechanisms be investigated to exploit such variability toward developing seed iron-dense cultivars. Using PIXE assay to investigate Fe distribution in seed tissues of *Phaseolus* species, Cvitanich et al. (2010, 2011) concluded that (1) the distribution of Fe in seed depends on the species and genotype, (2) high concentrations of Fe accumulate in cells surrounding the provascular tissue, (3) the tissue in the proximity of the provascular bundles holds up to 500 $\mu g\, g^{-1}$ Fe, depending on genotypes, and (4) the largest proportion of seed Fe in *Phaseolus* species is stored in compounds and cell parts different from ferritin and starch vacuoles. These results indicate that more studies are needed to assess the patterns of micronutrient distribution in seeds, and that micronutrient distribution criteria should be integrated into the selection strategies for biofortification of staple crops.

In summary, CGIAR and national agricultural research systems (NARS) institutions hold large collection of germplasm, both cultivated and wild relatives' of cereal and legume crops. The core or mini-core collections available in these crops may be used to identify seed mineral-dense germplasm. The germplasm from regions deficient in soil micronutrients should receive priority for evaluation as such germplasm are expected to develop inherent adaptation mechanisms that favor enhanced nutrient acquisition, transport, distribution, and relocation in plants/seeds. Few germplasm lines with high seed-Fe and/or -Zn concentrations have been reported in common bean, maize, pearl millet, rice, sorghum, and wheat. Wild and weedy relatives of common bean and wheat have shown abundant variability for Fe and Zn. Mutants with moderate-to-high reduction in phytate are available in

barley, common bean, maize, rice, soybean, and wheat. Genotypic differences in iron bioavailability have been reported in common bean, maize, rice, and wheat, which should be further explored. Several methods are available with high precision to map elements distribution, which may be used to identify barriers to Fe and Zn accumulation in the seed.

VI. EXPLOITING NATURAL GENETIC VARIATION TO BREED FOR SEED MINERAL-DENSE CULTIVARS

A. Fixing the Biologically Attainable Target to Breed for Seed Mineral-Dense Crops

Several factors must be taken into consideration when setting the target levels for enhancing the nutritional status of food crops by breeding. These include (1) mapping the human populations with micronutrient deficiency, (2) food habits of those suffering from micronutrient malnutrition, (3) the major staple crops grown in micronutrient-deficient regions and their nutrient profiles, (4) the recommended micronutrient requirement vis-à-vis daily nutrient intake, (5) the genetic variation for micronutrients in germplasm pools and cultivars/hybrids produced in the region or of possible production there, and (6) the bioavailability, bioconversion, and bioaccessibility of the micronutrients in the crop or combination of crops consumed in the diet (Nestel et al. 2006; Ortiz-Monasterio et al. 2007; Pfeiffer and McClafferty 2007; Bouis and Welch 2010). The target set for crop biofortification for one nutrient may not be the same for a different micronutrient, and may further differ from one country or region to another. The baseline data of daily intake of minerals may vary as detected for iron concentration in India. The intake of iron in India is less than 50% of the recommended dietary allowance, and iron density is about 8.5 mg 1000 $Kcal^{-1}$, with significant differences in absolute amounts among regions. Diets in Indian state of Andhra Pradesh with rice as staple have lowest iron (7 mg 1000 $Kcal^{-1}$), while diets in Gujarat and Madhya Pradesh with pearl millet as the staple have the highest iron intake (16 mg 1000 $Kcal^{-1}$) (Nair and Iyengar 2009). Likewise, several target regions for crop biofortification (Fe, Zn, and provitamin A) interventions have been identified in Latin America and the Caribbean (Zapata-Caldas et al. 2009). For example, interventions in northern Colombia appear promising for all crops, while sites for bean biofortification are widely scattered throughout the country. The most promising sites in Nicaragua are found in the

center-north region, while candidate sites for biofortification in Bolivia are found in the central part of the country and in the Andes Mountains. Poverty levels indicated that northeast Brazil is the most important region for biofortification in that part of South America.

Variations in the conversion factor of provitamin A (β-carotene) to vitamin A (retinol) in food crops should also need to be considered when defining breeding targets for β-carotene (Tang 2010). Significant genetic variation for seed-Fe and -Zn concentrations has been reported for cereal and legume crops, with some genotypes having more bioavailable seed micronutrients than others (see Section V.A). Most modern cultivars/hybrids have lower micronutrients per se than those reported in germplasm pools of a given species (Graham et al. 1999; Frossard et al. 2000). The adverse effects of processing, storage, and cooking on nutrient concentrations losses are known (see Section III.B.2). In addition, there are certain elements present in the seed that either act as enhancers (i.e., ascorbic acid) or inhibitors (i.e., phytase) of micronutrient uptake and absorption (see Section III.B.1).

All these variables need to be factored in when setting the breeding targets for improved nutritional quality of food crops. HarvestPlus has set the tentative breeding targets for improving the micronutrient density of several food crops. For example, the tentative targets to increase seed-Fe concentration of rice, wheat, pearl millet, common beans, maize, cassava, and sweet potato are 15, 59, 88, 107, 60, 45, and 85 $\mu g\, g^{-1}$ on a dry weight basis, respectively, while those for Zn are fixed at 28, 38, 66, 56, 38, 34, and 70 $\mu g\, g^{-1}$ (Bouis and Welch 2010).

For provitamin A, the targets set are 17, 17, 23, 34, 17, 48, and 91 $\mu g\, g^{-1}$ for rice, wheat, pearl millet, common bean, maize, cassava, and sweet potato, respectively (Bouis and Welch 2010). Such an approach can be applied to define the target levels for other micronutrients as well. However, targets should be dynamic depending on the severity of the micronutrient deficiency and the progress realized through breeding for developing mineral-dense cultivars/hybrids.

B. Genotype × Environment Interaction and Relationships Between Seed Minerals and Agronomic Traits

Knowledge of the effects of G, environments (E), and genotype × environment interaction (GEI) is important for developing nutritionally enhanced crop cultivars. Like yield and yield attributing traits, seed-Fe and -Zn concentrations in common bean, maize, rice, and wheat are influenced by location (or E), G, and GEI (Table 3.2), with location effects generally much larger than those of either G or GEI effects. The

Table 3.2. Environment (location) and genotype (G) × environment (E) interaction effects on seed-iron (Fe) and -zinc (Zn) content in common bean, maize, rice, and wheat covering a period from 2003 to 2010.

Fe			Zn			
Genotype	Environment	G × E interaction	Genotype	Environment	G × E interaction	References
Common bean						
***	***	*	***	***	NS	Astudillo and Blair 2008
Maize						
* (15%)	** (31%)	* (28%)	** (15%)	*** (26%)	** (35%)	Oikeh et al. 2004b
	NS	**		NS	**	Oikeh et al. 2003b
Rice						
	*	NS				Abilgos-Ramos et al. 2004
Wheat						
*** (9%)	*** (71%)	*** (20%)	*** (19%)	*** (65%)	*** (16%)	Gómez-Becerra et al. 2010
*** (10%)	*** (86%)	*** (3%)	*** (3%)	*** (89%)	*** (8%)	Zhang et al. 2010
NS	** (99%)	NS	NS	** (92%)	NS	Joshi et al. 2010
*** (31%)	*** (9%)	*** (54%)	*** (34%)	*** (4%)	*** (51%)	Ficco et al. 2009
* (50%)	** (10%–51%)	(18%–48%)	* (9%)	** (32%–84%)	(7%–49%)	Morgounov et al. 2007
* (1%)	*** (98%)	NS	* (1%)	*** (98%)	NS	Distelfeld et al. 2007

Notes: *, **, and *** indicate significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Figures in the parenthesis refer to % phenotypic variation. Ns, nonsignificant

growing environments had no effect on bioavailable Fe in maize (Oikeh et al. 2004a); however, Pixley et al. (2011a) detected larger E than GEI for Fe bioavailability in maize. It is therefore suggested that responses of cultivars to different production environments need to be well understood to improve the probability of predicting and identifying cultivars that are not only high in seed-Fe and/or -Zn concentrations but also these micronutrients are more bioavailable to absorption (Briat and Lobreaux 1997; Pixley et al. 2011a).

The environmental variables such as pH, temperature, solar radiation, precipitation, organic matter, and soil texture have the potential to influence nutrient concentration (Tisdale and Nelson 1975; Römheld and Marschner 1986; Cabuslay et al. 2003; Abilgos-Ramos et al. 2004; Joshi et al. 2010) and must be taken into consideration while explaining the variation for seed micronutrients in germplasm or when assessing the nutritional quality of staple food crops grown in diverse agroecological conditions.

Character association between seed mineral concentrations may indicate the existence of one or more common genetic-physiological mechanisms involved in mineral uptake by the root system, translocation, and redistribution within the plant tissues, remobilization to the seed, and accumulation in the developing seed (Chatzav et al. 2010). Both mineral concentration (amount per unit weight, i.e., $mg\,kg^{-1}$) and mineral content (amount per seed, i.e., $\mu g\ seed^{-1}$) are positively correlated (Cakmak et al. 2004; Hacisalihoglu et al. 2005; Stangoulis et al. 2007) and either can be used to estimate the quantity of the minerals in the seeds (see Section V.A).

An understanding of the nature of association between different minerals and also with seed yield and seed weight (100 or 1,000-seed weight) should facilitate the selection of mineral-dense progenies in breeding. The published evidence suggests that seed-Fe and -Zn concentrations, in most cases, are highly significant and positively correlated in common bean, pearl millet, rice, and wheat (Table 3.3), which suggests that genes for Fe and Zn accumulation cosegregate or are pleiotropic. Such relationships could be exploited toward selecting progenies with high seed minerals in the segregating populations. Further studies revealed that Fe and Zn in the flag leaves of *Aegilops* species are highly significant and positively correlated with seed Fe and Zn (Rawat et al. 2009a,b); however, such relationships were not found in common bean (Tryphone and Nchimbi-Msolla 2010). More studies are needed to elucidate these relationships prior to using flag leaf for early selection of plants with potentially high seed Fe and Zn in breeding programs.

Table 3.3. Relationships among seed-iron (Fe) and -zinc (Zn) contents in common bean, maize, pearl millet, rice, sorghum, and wheat covering a period from 2003 to 2010.

Number and type of experimental material	Correlation coefficient	References
Common bean		
90 Genotypes from Tanzania	0.416**	Tryphone and Nchimbi-Msolla 2010
110 RILs involving G14519 and G4825, three sites	0.483**, 0.636***, 0.686***	Blair et al. 2010a
87 RIL involving G19833 and DOR364, two sites, two methods	0.594**–0.751**	Blair et al. 2009b
10 Cultivars	0.75***	Oomah et al. 2008
40 Colombian varieties, low and high P soils	0.59**, 0.75***	Astudillo and Blair 2008
76 RILs involving Jamapa and Calima	0.60**	Hacisalihoglu et al. 2005
24 Germplasm accessions	0.65*	Welch et al. 2000a
Maize		
294 F_4 lines and six controls including parental lines; two environments	0.13**–0.14**	Šimic et al. 2009
21 Late-maturing varieties and 3 locations	0.48***	Oikeh et al. 2004a
49 Late-maturing tropical maize and 3 environments	0.51***	Oikeh et al. 2003
Pearl millet		
61 Genotypes	0.88**	Selvi and Rajarathinam 2009
54 S_1 progenies from two populations	0.80**–0.82**	Gupta et al. 2009
120 Lines including populations and population progenies, germplasm, seed parents, and pollinators	0.84**	Velu et al. 2007
79 Lines including male steriles, testers, and hybrids	0.56**	Arulselvi et al. 2007
Rice		
202 Traditional and modern cultivars	0.346**	Anandan et al. 2011
129 Double haploid lines involving IR64 and Azucena	0.71***	Stangoulis et al. 2007

(*continued*)

Table 3.3 (*Continued*)

Number and type of experimental material	Correlation coefficient	References
Sorghum		
29 Core collection accessions	0.75**	Kumar et al. 2009
84 Including hybrid parental lines, germplasm, cultivars, and those differing in seed quality traits	0.55**	Reddy et al. 2005
Wheat		
600 Core collection accessions of diverse origin	0.81***	Velu et al. 2011
154 Genotypes including wild species and 2 environments	0.40***–0.67***	Chatzav et al. 2010
265 Lines including leading cultivars and advanced lines	0.75***	Zhang et al. 2010
152 RILs involving durum and wild emmer wheat, 3 environments	0.79***	Peleg et al. 2009
90 Double haploid lines, tested at 2 locations for 2 years	0.55*–0.84*	Genc et al. 2009
175 Diverse lines involving bread (winter/spring), durum, spelt, einkorn, and emmer wheat's	0.29**	Zhao et al. 2009
19 Wild emmer germplasm, and 5 environments	0.50**	Gómez-Becerra et al. 2010
66 Winter/spring wheat cultivars and improved lines and 5 locations	0.79***	Morgounov et al. 2007

Abbreviations: RIL = recombinant inbred line population individuals.

Highly significant and positive correlations (0.82–0.99) between seed P and phytate have been reported for common bean, pearl millet, and rice (Lolas and Markakis 1975; Stangoulis et al. 2007; Selvi and Rajarathinam 2009), while a low but positive and significant association was found for seed phytate with Fe and Zn in common bean (Cichy et al. 2009). Further, several studies in wheat and common bean revealed a moderate but positive and significant association of seed P with Fe (0.42–0.55) and Zn (0.46–0.63) (Gelin et al. 2007; Peleg et al. 2009; Zhao et al. 2009; Zhang et al. 2010). The implications of the above are that while it should be possible to breed for high seed-Fe and -Zn concentrations with reduced phytate concentration in pearl millet, this may not be possible in wheat or common bean.

In yet another issue of correlations and micronutrient versus macronutrient concentrations, seed yield is significantly and positively associated with seed weight in cereals and legumes (Upadhyaya et al. 2002; García del Moral et al. 2003; Upadhyaya 2003; Maman et al. 2004; Morgounov et al. 2007). However, it is either not associated with seed-Fe and -Zn concentrations in pearl millet or shows a low but significant negative association in wheat (Morgounov et al. 2007; Peleg et al. 2009; Zhao et al. 2009) and positive association in common bean (Gelin et al. 2007). A negative association may pose problems for breeding of seed mineral-dense cultivars with high seed yield per se. Seed weight in pearl millet is highly significant and positively associated with seed Fe ($r=0.80$) and Zn ($r=0.85$) (Velu et al. 2007), while it is significant and positively correlated ($r=0.61$) with Fe in common bean (Gelin et al. 2007).

Another question is whether there are any relationships between seed-Fe (or -Zn) concentration and bioavailability. Limited studies on seed-Fe and -Zn concentration and bioavailability in maize, rice, and wheat revealed no such associations (Glahn et al. 2002; Oikeh et al. 2003a,b,2004a), indicating that it is possible to significantly increase both concentration and bioavailability of either Fe or Zn in the seed by breeding and selection.

C. Quantitative Trait Loci (QTL) Associated with Seed Iron, Zinc, and Phytate Concentrations

Genomics science since the 1990s has made phenomenal advances toward developing a large number of molecular markers and genetic linkage maps allowing the mapping and/or cloning of QTL and identification of candidate gene(s) associated with agriculturally beneficial traits, which can lead marker-assisted selection (MAS)

for desirable genes in crop breeding (Dwivedi et al. 2007; Collard and Mackill 2008). Automation of high-throughput assays including next generation sequencing technologies and associated data mining tools provide breeders/molecular biologists opportunities to handle and interpret large data sets (Varshney et al. 2009; Feuillet et al. 2010). Furthermore, the genomes of agriculturally important crops such as maize, rice, sorghum, and soybean have been sequenced (IRGSP 2005; Paterson et al. 2009; Schanable et al. 2009; Schmutz et al. 2010), while several projects under way to sequence genomes of many other agriculturally important food crops (Feuillet et al. 2010). The deoxyribonucleic acid (DNA) sequence variants across species or among strains within a species may be used as new genetic tools for developing markers and subsequently crop cultivars with specific characteristics.

The crops included in this review have abundant genetic resources to dissect population structure and diversity in germplasm collections to identify genetically diverse germplasm with beneficial traits. However, only recently have DNA marker-based technologies been used to identify QTL associated with increased seed-Fe and -Zn concentrations in barley, common bean, pearl millet, rice, and wheat, revealing many QTL with varying effects; some with major phenotypic variation while many others with minor effects (Table 3.4). For example, in common bean a QTL on linkage group (LG) b09 was found for Zn by Gelin et al. (2007), while QTL found by Cichy et al. (2009) on LG b01 (near the *fin* gene) accounted for 34% of variation for seed-Fe and -Zn concentrations and also overlapped with a major QTL (19% variation) for increased seed phosphorus (P) concentration. Finally, other QTL on LG b06 accounted for 36% variation for seed Fe and 39% variation for seed Zn that same study (Cichy et al. 2009). This latter QTL was linked with a QTL found for Mesoamerican beans by Blair et al. (2010c). Further studies by Blair et al. (2009b, 2010b) in both inter- and intragene pool populations, respectively, found specific major and minor QTL for Fe and Zn concentrations with the former type mainly on LG b11. Therefore, at least four major QTL have been identified in common bean affecting micronutrient concentration depending on the gene pool and genetic background of the material tested.

In other crops, meanwhile, a QTL for seed-Zn on chromosome 7A mapped at *Xcfd31–Xcfa2049* explained 19% variation in wheat (Tiwari et al. 2009). In rice, a seed-Zn QTL mapped at *RM235–RM17* on chromosome 12 accounted for 13% variation and colocated with seed-Fe QTL that mapped at *RM270–RM17* and accounted 14%

Table 3.4. Summary of marker/QTL analysis of seed iron (Fe) and/or zinc (Zn) concentration in barley, common bean, rice, and wheat covering a period from 2003 to 2011.

QTL summary	References
Barley	
Sequence-specific PCR-based dominant marker, SZnR1, located on the short arm of chromosome 2H, associated with high seed zinc concentration and content	Sadeghzadeh et al. 2010
Three of the five most favorable QTL alleles increased seed-Zn concentration and content by an average of 53% and 75%, respectively	Lonergan et al. 2009
Common bean	
Total of nine seed mineral QTL were identified in an Andean × Andean mapping population on five linkage groups (LGs) with the most important being new loci on b02 and other QTL on b06, b08, and b07 near phaseolin. Seed weight QTL were associated with these on b02 and b08	Blair et al. 2011
A set of across site, overlapping iron and zinc QTL were discovered for a Mesoamerican × Mesoamerican mapping population on LG b06 suggesting a possibly pleiotropic locus and common physiology for mineral uptake or loading. Other QTL for mineral concentration or content were found on LGs b02, b03, b04, b07, b08, and b11 and together with the b06 cluster were mostly novel compared to loci found in previous studies of the Andean gene pool or inter-gene pool crosses	Blair et al. 2010c
Total of 26 QTL were identified in an inter-gene pool mapping population for the mineral × trial × method combinations of which half were for iron concentration and half for zinc concentration. Many of the QTL (11) for both iron (5) and zinc (6) clustered on the upper half of LG B11, explaining up to 47.9% of phenotypic variance, suggesting an important locus useful for marker-assisted selection. Other QTL were identified on LG B3, B6, B7, and B9 for zinc and B4, B6, B7, and B8 for iron	Blair et al. 2009a
Fe: 11 QTLs on six LGs, 8%–36% variation; Zn: 11 QTL on four LGs, 9%–39% variation; a QTL on LG B1 (nearest to *fin* marker) accounted 34% variation for Fe and Zn, while another QTL on LG B6 (nearest to *AGAT05* marker) accounted 36%–39% variation for Fe and Zn	Cichy et al. 2009
A locus on LG9 associated with Zn accumulation	Gelin et al. 2007
Two QTL for Fe (25% variation) and one QTL for Zn (15% variation)	Guzmán-Maldonado et al. 2003

(*continued*)

Table 3.4 (*Continued*)

QTL summary	References
Rice	
Two QTL for Fe on chromosome 2 and 9 and three QTL for Zn on chromosome 5, 8, and 12; a major QTL for Zn on chromosome 8 accounted 11%–19% variation	Garcia-Oliveira et al. 2009
Three QTL for Fe on chromosomes 2, 8, and 12, while two QTL for Zn on 1 and 12; a common QTL for Fe and Zn on chromosome 12 accounted 13%–14% variation	Stangoulis et al. 2007
Wheat	
Two QTL for seed Fe on chromosomes 2A and 7A, *QFe.pau-2A* and *QFe.pau-7A* explaining 12%–13% variation, and one QTL for seed Zn on chromosome 7A, QZn.pau-7A explaining 19% variation	Singh et al. 2010a
Two QTLs for Fe on chromosomes 2A and 7A, mapped at *Xwmc382–Xbarc124* and *Xgwm473–Xbarc29*, explained 12%–13% variation; a QTL for Zn on chromosome 7A, mapped at *Xcfd31–Xcfa2049*, explained 19% variation	Tiwari et al. 2009
A QTL on chromosome 3D for seed-Fe and four QTLs on chromosomes 3D, 4B, 6B, and 7A for Zn concentrations; QTL for seed-Fe concentration colocated with a QTL for shoot-Fe concentration and seed weight, with alleles for high Fe concentration coming from the same parent	Genc et al. 2009
11 QTLs on chromosomes 2A, 5A, 6B, 7A, and 7B for Fe and 6 QTL on chromosomes 2A, 2B, 3A, 4B, 5A, 6A, 6B, 7A, and 7B for Zn; clusters of QTLs on chromosome 2A, 5A, 6B, and 7A for seed protein and minerals	Peleg et al. 2009
Four QTLs for Zn concentration and seven QTLs for Zn content, with concentration QTLs colocated with those of content QTLs, possible to improve both traits simultaneously	Shi et al. 2008

phenotypic variation (Stangoulis et al. 2007). In barley, specific markers have been developed for a high zinc QTL on chromosome 2H (Sadeghzadeh et al. 2010), while other QTLs have been identified by Lonergan et al. (2009).

Iron reductases are members of the protein super-family of flavocytochromes and function in roots to convert Fe from a plant unavailable form (ferric, Fe^{3+}) to an available form (ferrous, Fe^{2+}) that can be readily absorbed (Grusak 1995). IRA is known to vary with plant growth conditions (e.g., soil pH and available iron concentration) (Grusak 2000). Common bean genotypes with high seed-Fe showed high IRA than those with low seed-Fe, suggesting a link between root uptake and seed loading of Fe in common bean (Grusak 1994, 2000, 2002). More recently, Blair et al. (2010b) reported a single major QTL for IRA under Fe-limited conditions (1 μM) on LG b02, and another major QTL under Fe-sufficient conditions (15 μM) on LG b11 that was associated with several QTL for seed Fe in common bean. Thus, the QTL for IRA under Fe-limited conditions may be useful in environments where beans are grown in alkaline soils, while the QTL for IRA under Fe-sufficient conditions may be useful for selecting for enhanced seed nutritional quality (Blair et al. 2010b).

Wild emmer wheat germplasm harbors a rich allelic diversity, including for seed minerals (Xie and Nevo 2008). A major locus, *Gpc-B1* (a 250-kb locus) mapped as a simple Mendelian locus (Distelfeld et al. 2006), associated with increased seed-protein (38%), -Fe (18%), and -Zn (12%) concentrations from wild emmer wheat germplasm (*Triticum dicoccoides*), encodes a NAC transcription factor (*NAM-B1*) that accelerates senescence and increases nutrient remobilization from leaves to developing seeds (Uauy et al. 2006; Distelfeld et al. 2007). *Triticum turgidum* is another useful wild emmer germplasm for improving seed mineral concentration in wheat. Peleg et al. (2009) mapped 82 QTL for 10 seed minerals (LOD score range of 3–17), with most of the positive alleles contributed by wild emmer accession, G18-16, and many QTL for the same trait mapped to homoeologous positions, reflecting synteny between the A and B genomes. *TtNAM-B1* affecting seed-protein, -Fe, and -Zn originating from wild emmer wheat has been cloned (Distelfeld and Fahima 2007). Furthermore, Singh et al. (2010a) identified two QTL (*QFe.pau-2A* and *QFe.pau-7A*) for Fe and a QTL (*QZn.pau-7A*) for Zn, which they transferred into interspecific progenies involving *Aegilops kotschyi* and *Aegilops peregrine*, both UUSS genome species. Such progenies showed 60%–136% enhanced seed-Fe and -Zn concentrations and 50%–120% increased Fe and Zn contents per seed as compared to the control cultivar that was introgressed with these

QTL (Tiwari et al. 2010). The profiling of introgression using simple sequence repeats (SSRs), genomic *in situ* hybridization (GISH), and fluorescent *in situ* hybridization (FISH) analysis further confirmed the introgression of chromosome 2S, 2U, 7S, and 7U into these progenies (Singh et al. 2010a). More recently, genetic mapping identified five putative QTL for seed-Fe density and two QTL for seed-Zn density in pearl millet (Kumar et al. 2010b).

A number of QTL for seed phosphorus (P) and/or phytate concentrations have been reported in common bean, rice, sorghum, soybean, and wheat (Table 3.5), some with either major effects or colocated with QTL affecting seed-Fe or -Zn concentration. For example, a major QTL for P (19% variation) on b01 colocated with QTL accounting 34% variation each for seed Fe and Zn in common bean (Cichy et al. 2009). Furthermore, QTL for seed P or phytate concentration or content related to seed weight QTL on LGs b06, b07, and b10 (Blair et al. 2009c) or genes coding for candidate enzymes involved in phytic acid synthesis pathway and markers associated with each gene (Fileppi et al. 2010) have been mapped in common bean. In rice, the candidate gene for low-phytate mutant alleles and markers (LPA1_CAPS for *lpa1-1* and LPA1_InDel for *lpa1-2*) showed complete cosegregation with mutant phenotypes (Zhao et al. 2008). Furthermore, the two QTL for seed-Zn concentration on chromosomes 4A and 4D colocated with QTL for P concentration, while four QTL for seed-Zn content on chromosome 2D, 3A, and 4A colocated with the QTL for P content, reflecting positive correlation between the seed-Zn and -P concentrations (see Section VI.B), which may provide opportunities for simultaneous improvement in seed-P and -Zn density in wheat (Shi et al. 2008).

Two QTL mapped onto LGs L and N control low phytate in soybean line, CX 1834 (Walker et al. 2006; Gao et al. 2008). Further, Saghai-Maroof et al. (2009) mapped and sequenced a putative multidrug resistance-associated protein (MRP) gene on LG N that contributes to low-phytate phenotype in CX 1834. This A to T mutation provided a single nucleotide polymorphism (SNP) marker for introgressing the low-phytate QTL from CX 1834 into desired breeding lines (Saghai-Maroof et al. 2009).

More recently, genes coding for candidate enzymes involved in the phytic acid pathway have been mapped and identified markers associated with each gene (*PvMIPSs*, *PvMIPSv*, *PvIMP*, *PvMIK*, *PvIPK2*, *PvITPKά*, *PvITPKβ*, *PvIPK1*), which may represent a useful resource to select genetic variants with low-phytate trait in common bean (Fileppi et al. 2010). Furthermore, González et al. (2010) discovered how phytate is produced in plants, by solving the structure of the protein $InsP_5$ 2-Kinase (IP_5 2-K), a distant member of the IPK family,

Table 3.5. Summary of marker/QTL analysis of seed phosphorus (P) and phytate (Phyt) concentration in barley, common bean, rice, sorghum, soybean, and wheat covering a period from 2005 to 2010.

QTL summary	References
Barley	
Flanking markers mapped 3 low phytic acid mutant alleles: *lpa1* at EBmac415 and Msu21; *lpa2-1* at Bmag120 and AWBMS0022; *lpa678* at EBmac701 and Bmag714B	Oliver et al. 2009
Common bean	
Genes coding for candidate enzymes involved in phytic acid pathway mapped and markers associated with each gene (*PvMIPSs, PvMIPSv, PvIMP, PvMIK, PvIPK2, PvITPKα, PvITPKβ, PvIPK1*) identified	Fileppi et al. 2010
Eight QTL for P on six LGs, in an Andean × Andean bush bean cross, 11%–40% variation; two QTL for Phyt on two LGs, 17%–18% variation; a QTL for P (19% variation) on B1 colocated with QTLs accounting 34% variation each for Fe and Zn on same LG	Cichy et al. 2009
Six QTL for P on LG B2 and B6 and three QTL for Phyt on LG B6, with QTL for P or Phyt were related to seed weight QTL on LGs B6, B7, and B10 in an inter-gene pool mapping population	Blair et al. 2009b
Rice	
Mutant alleles of the low phyt homozygous lethal (*XS-lpa2-1*) and nonlethal (*XS-lpa-2-2*) mapped: *XS-lpa2-1* gene to a region on chromosome 3 between marker RM14360 and RM1332, where also located the rice orthologue (*OsMRP5*) of the maize *lpa1*; a single base pair change in the sixth exon of *XS-lpa2-1* and a 5-bp deletion in the first exon of *XS-lpa2-2* resulted these mutations	Xu et al. 2009
TIGR locus LOC_Os02g57400 identified as the candidate gene for mutant alleles: *lpa1-1* is a single base pair substitution mutation while *lpa1-2* involves a 1,475 bp fragment deletion; a CAPS marker (LPA1_CAPS) for *lpa1-1* and an InDel marker (LPA1_InDel) for *lpa1-2* confirmed complete segregation with LPA phenotypes	Zhao et al. 2008
A single base pair change resulted *lpa* mutant, N15-186, mapped at RM15875 and RM15907 on chromosome 3, which also harbor rice orthologue of maize *lpa3*; *lpa* N15-186 is a mutant allele of the rice *myo*-inositol kinase (*Os MIK*) gene	Kim et al. 2008

(*continued*)

Table 3.5 (*Continued*)

QTL summary	References
A common QTL for P and Phyt on chromosome 5, explained 24% variation	Stangoulis et al. 2007
Lpa1 locus (135 kb) fine mapped at RM3542 and RM482 and further delimited to a 47 kb region containing eight putative open reading frames	Andaya and Tai 2005
Soybean	
A locus on LG N near *Satt237* accounted for 41% variation in Pi, which is inversely related with Phyt, while another locus near *Satt237* on LG L explained 11% variation and interaction between the two loci accounted additional 8%–11% variation	Walker et al. 2006
Wheat	
Four QTL for P concentration and six QTL for P content; two QTL for Zn concentration on chromosomes 4A and 4D colocated with the QTLs for P concentration, while four QTL for Zn content on 2D, 3A, and 4A colocated with QTLs for P contents	Shi et al. 2008

which represents a key point in the metabolism of highly phosphorylated inositols, and could be a valuable tool in plant physiology to design low-phytate crops.

D. QTL Mapping, Cloning, and Introgression of β-Carotene into Adapted Germplasm

The natural plant pigments, termed as carotenoids, which are fat soluble, are important source of vitamin A and antioxidants. The two groups of carotenoids are provitamin A and nonprovitamin A carotenoids. The former includes three carotenoids, namely β-carotene, β-cryptoxanthin, and α-carotene, all precursor for vitamin A, while the latter consists of lutein and zeaxanthin, which act as antioxidants. Of all the carotenoids, β-carotene is the most widely distributed in plants and the one most efficiently converted to vitamin A. The consumption of carotenoid-rich foods is associated with reduced risks of developing cancer and cardiovascular diseases, enhanced immune responses, improved vision, and prevention of night blindness as well the maintenance of healthy skin and gastrointestinal or respiratory systems. Carotenoids in plants play a crucial role in photosynthesis, membrane stability, growth, and development (Menkir et al. 2008).

Unlike other agronomically important traits, only limited germplasm sets have been assessed for β-carotene, predominantly in chickpea, maize, pearl millet, sorghum, and wheat (Table 3.6), with some lines accumulating β-carotene as high as 7.6 $\mu g\, g^{-1}$ in pearl millet (Hash et al. 1997) and 15.6 $\mu g\, g^{-1}$ in maize (Harjes et al. 2008; Menkir et al. 2008). Genotypic differences for β-carotene have also been reported in rice: some germplasm had shown β-carotene in unpolished seeds while others having no β-carotene in unpolished seeds. The rice germplasm with β-carotene in unpolished seeds include Amarillo Cuba, Dudemasino, Sirendah Kuning, Bongkitan, Calibo, Khao Dawk Mali 105, and Klemas (Tan et al. 2005). Furthermore, Kandlakunta et al. (2008) in a comprehensive study involving major cereals and legumes and commonly consumed vegetables detected high β-carotene in chickpea (15.7 $\mu g\, g^{-1}$), green gram (12.8 $\mu g\, g^{-1}$), red gram (12.4 $\mu g\, g^{-1}$), and maize (17.1 $\mu g\, g^{-1}$), while among vegetables, they detected high β-carotene in yellow pumpkin (118 $\mu g\, g^{-1}$), green chillies (102 $\mu g\, g^{-1}$), field beans (55.4 $\mu g\, g^{-1}$), French bean (39.3 $\mu g\, g^{-1}$), ridge gourd (32.4 $\mu g\, g^{-1}$), green beans (23.9 $\mu g\, g^{-1}$), and brinjal (16.9 $\mu g\, g^{-1}$).

Maize is an important source of provitamin A (β-carotene, α-carotene, and β-cryptoxanthin) and the nonprovitamin A including lutein and zeaxanthin. Most of the yellow maize grains consumed worldwide have

Table 3.6. Natural genetic variation for β-carotene in chickpea, maize, pearl millet, sorghum, and wheat covering a period from 1962 to 2009.

No. of germplasm evaluated	Summary of the variation in β-carotene concentration ($\mu g\, g^{-1}$ seed)	Range variation in β-carotene concentration ($\mu g\, g^{-1}$ seed)	References
Chickpea			
Two lines	0.09–0.48	0.37	Abbo et al. 2005
Maize			
155 Inbreds	0.13–1.98	1.85	Yang et al. 2010
228 Inbred	0.06–13.6	13.54	Harjes et al. 2008
233 RILs and parental lines	0.05–2.04	1.99	Chander et al. 2008
421 Yellow endosperm inbreds	0.3–15.6	15.3	Menkir et al. 2008
17 Tropical varieties	0.45–2.18	1.73	Menkir and Maziya-Dixon 2004
10 Inbreds	0.56–2.40	1.84	Egesel et al. 2003
125 Inbreds	From traces to 7.3	7.3	Quackenbush et al. 1963
109 Yellow endosperm lines	0.5–5.1	4.6	Brunson and Quackenbush 1962
Pearl millet			
IP 15533 and IP15336	6.1–13.7	7.6	Hash et al. 1997
13 Lines	0.23–0.63	0.4	Khangura et al. 1980
Sorghum			
10 Carotenoids diversity panel accessions	0.10–0.22	0.12	Salas Fernandez et al. 2009
KS115 and Macia	0.025–0.074	0.049	Salas Fernandez et al. 2008
RILs (KS115 × Macia)	0.003–0.148	0.145	
82 Including yellow endosperm lines	0.56–1.13	0.57	Reddy et al. 2005
10 Lines	0.22–3.23	3.01	Worzella et al. 1965
Wheat			
13 Lines	0.03–o.13	0.10	Ramachandran et al. 2010
5 Lines	3.0–8.5a	5.5[z]	Santra et al. 2005

[z]Part per million.

only 0.5–1.5 $\mu g\,g^{-1}$ β-carotene. Maize has been extensively studied for molecular polymorphism, mapped QTL, chemical pathways, and genes involved in carotenoids biosynthesis, including β-carotene (Table 3.7). Carotenes are intermediates in the carotenoids biosynthetic pathway and lycopene (the immediate precursor of provitamin A carotenes) represents a branch point in this pathway, which is further modified by lycopene β-cyclase and lycopene ε-cyclase (LCYE) enzymes that catalyze formation of terminal β- and ε-rings, respectively, to form either β-carotene, α-carotene, or β-cryptoxanthin. This pathway continues with hydroxylation of the carotenes that depletes the provitamin A pool by converting these compounds to nonprovitamin A xanthophylls. Pathway branching and hydroxylation are therefore key determinants in controlling vitamin A levels. Polymorphism at the *LCYE* locus in maize explained 58% of the variation in α- and β-carotene and a threefold difference in provitamin A compounds (Harjes et al. 2008), while a rare genetic variant, *β-carotene hydroxylase 1* (crtRB1), increases β-carotene substantially in maize grains (Yan et al. 2010). Further, metabolite sorting of a germplasm collection identified 10 genetically diverse subsets representing biochemical extremes for maize kernel carotenoids and transcript profiling of this subset led to the discovery of the *Hydroxylase 3* locus that coincidently mapped to a carotene QTL (Chander et al. 2008). The natural alleles at *Hydroxylase 3* locus contribute 78% of variation and approximately 11-fold differences in β-carotene relative to β-cryptoxanthin and 36% of the variation and fourfold difference in absolute levels of β-carotene (Harjes et al. 2008). The reduction in *HYD3* transcripts leads to reduced conversion of β-carotene to downstream xanthophylls, causing β-carotene to accumulate (Vallabhaneni et al. 2009). Genetics tests such as the *HYD3* assay (Vallabhaneni et al. 2009) together with the previously described *LCYE* assay (Harjes et al. 2008) may be used to select germplasm containing optimal *HYD3* and *LCYE* alleles in breeding programs, which will lead to higher β-carotene levels in maize endosperm when both genes are highly expressed than either with optimal alleles of either gene alone. Furthermore, the experimental evidence from the association and linkage mapping reveals that *crtRB1* underlies a principal QTL associated with β-carotene concentration and conversion in maize kernels and *crtRB1* alleles associated with reduced transcript expressions correlate well with higher β-carotene (Yan et al. 2010). The most favorable *crtRB1* alleles that are rare in frequency and unique to temperate germplasm are being introgressed via inexpensive PCR-marker-assisted selection into tropical maize germplasm adapted to developing countries. A program at CIMMYT has already achieved

Table 3.7. QTL associated with β-carotene in chickpea, maize, and sorghum covering a period from 2004 to 2010.

QTL summary	Reference
Chickpea	
Four QTL associated with β-carotene	Abbo et al. 2005
Maize	
A gene encoding β-carotene hydroxylase 1 (*crtRB1*) underlies a major QTL associated with β-carotene concentration and conversion; *crtRB1* alleles associated with reduced transcript expression correlated with higher β-carotene concentration	Yan et al. 2010
Cloned/characterized four cDNAs encoding carotenogenic enzymes, two encoding CRTISO (*ZmCRISO1* and *ZmCRISO2*) and two encoding BCH (*ZmBCH1* and *ZmBCH2*), mapped on different chromosomes; all four genes expressed during endosperm development and mRNA levels increased with carotenoids accumulation until 25DAP except for *Zmcrtiso2* mRNA levels which remains high for another 5 days while the carotenoids content continues to increase	Li et al. 2010a
Hydroxylase3 locus mapped to a carotene QTL, with three alleles contributed 78% variation and 11-fold differences in β-carotene relative to β-cryptoxanthin and 36% variation and fourfold difference in absolute β-carotene	Vallabhaneni et al. 2009
31 QTL for total carotenoids on seven chromosomes, much of the variation explained by two loci *y1* and *y9*; candidate gene *phytoen synthase 1* (*psy1*) marker "Y1ssr" tightly linked to a major QTL explaining 7%–27% variation for carotenoids	Chander et al. 2008
Variation at *lycopene epsilon cyclase* (*lcyE*) locus alters flux down α-carotene versus β-carotene branches of the carotenoids pathway, with four natural *lcyE* polymorphism explained 58% of the variation in α-carotene and β-carotene and threefold difference in provitamin A compounds	Harjes et al. 2008
A major QTL for both β-carotene and β-cryptoxanthin on chromosome 6 and 7; genes *y1*, associated with phytoene synthase, and *vp9*, associated with ζ-carotene desaturase, linked with observed variation in carotenoids in maize and also with carotenoids variation in Solanaceae	Wong et al. 2004
Sorghum	
Color QTL significantly correlated with levels of all carotenoids, and color QTL colocalized with carotenoids QTL; β-carotene QTL (*Bc-1.1*, *Bc-2.1*, *Bc-2.2*, *Bc-2.3*, *Bc-10b.1*) mapped on chromosomes 1, 2, and 10 explained 8%–15% variation, with *Bc-2.2* (11.6% variation) remained stable across environments, and located close to *Psy3* gene involved in carotenoid biosynthesis pathway	Salas Fernandez et al. 2008

HarvestPlus provitamin A target concentrations by introgressing favorable *crtRB1* and *lcyε* alleles into tropical maize germplasm in a number of breeding lines, including a high β-carotene (15 $\mu g\,g^{-1}$) temperate hybrid, CI7 × DEexp that contains the most favorable *crtRB1* alleles (Yan et al. 2010; Pixley et al. 2011b). Several hybrids with β-carotene concentration between 5 and 8 $\mu g\,g^{-1}$ and agronomically competitive with commercial hybrids were tested during summer 2009/2010 in Zambia and Zimbabwe, and the best hybrids will be further evaluated in Mexico, Zambia, and Zimbabwe prior to their release in these countries (Pixley et al. 2011b). Likewise, the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, has introduced high β-carotene trait from temperate maize to tropical maize inbred lines, which ranged from 2.5 μg^{-1} to 10.5 μg^{-1}, and hybrids involving some of these inbreds showed 25%–79% more provitamin A concentration than Oba Super II, a commercial yellow hybrids widely grown in Nigeria. The grain yield and agronomic traits of the best hybrids were comparable to those of Oba Super II (IITA annual report, 2009/2010; http://annualreport.iita.org/?p=481). The University of Illinois, USA has also reported some of the high provitamin A lines of maize that include A 619, C 17, DE 3, and SC 55 (see supplementary Table 1, Yan et al. 2010). The researchers at Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora, India and other affiliated Indian Council of Agricultural Research (ICAR) institutions in India have identified few promising maize inbred lines (CM 136 and CM 138, 08 HPLET-03-8 and 08 HPLET-03-41, NAI 125, BAJIM-8-10 and SE 547), with total carotenoids ranging from 20.2 to 56.1 $\mu g\,g^{-1}$ seed and β-carotene from 10.6 to 14.9 $\mu g\,g^{-1}$ seed (P.K. Agrawal, VKPAS, pers. commun.).

The 'Golden Rice 2' genetic stocks have been used to introgress high β-carotene trait into several Asian rice cultivars, both *japonica* and *indica* types, and it is expected that the products from such introgressions will soon be available for national release in Bangladesh, India, Indonesia, and the Philippines (Barry 2011).

Sorghum landraces have shown significant variation for carotenoids, with lutein, zeaxanthin, and β-carotene the predominant carotenoids. Yellow-endosperm color QTL in sorghum colocalized with carotenoid QTL, with major β-carotene QTL *Bc-2.2* found close to *Psy3* gene, which is significantly associated with β-carotene concentration and endosperm color (Salas Fernandez et al. 2008). Furthermore, Salas Fernandez et al. (2009) reported that 164 yellow endosperm landraces from Niger and Nigeria clustered separately from the genotypes in a 68 individual diversity panel (Casa et al. 2008) with accessions differing in geographic origin and carotenoids content, which may provide

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10 Lines	0.22–3.23	3.01	Worzella et al. 1965
Wheat			
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only 0.5–1.5 $\mu g\, g^{-1}$ β-carotene. Maize has been extensively studied for molecular polymorphism, mapped QTL, chemical pathways, and genes involved in carotenoids biosynthesis, including β-carotene (Table 3.7). Carotenes are intermediates in the carotenoids biosynthetic pathway and lycopene (the immediate precursor of provitamin A carotenes) represents a branch point in this pathway, which is further modified by lycopene β-cyclase and lycopene ε-cyclase (LCYE) enzymes that catalyze formation of terminal β- and ε-rings, respectively, to form either β-carotene, α-carotene, or β-cryptoxanthin. This pathway continues with hydroxylation of the carotenes that depletes the provitamin A pool by converting these compounds to nonprovitamin A xanthophylls. Pathway branching and hydroxylation are therefore key determinants in controlling vitamin A levels. Polymorphism at the *LCYE* locus in maize explained 58% of the variation in α- and β-carotene and a threefold difference in provitamin A compounds (Harjes et al. 2008), while a rare genetic variant, *β-carotene hydroxylase 1* (crtRB1), increases β-carotene substantially in maize grains (Yan et al. 2010). Further, metabolite sorting of a germplasm collection identified 10 genetically diverse subsets representing biochemical extremes for maize kernel carotenoids and transcript profiling of this subset led to the discovery of the *Hydroxylase 3* locus that coincidently mapped to a carotene QTL (Chander et al. 2008). The natural alleles at *Hydroxylase 3* locus contribute 78% of variation and approximately 11-fold differences in β-carotene relative to β-cryptoxanthin and 36% of the variation and fourfold difference in absolute levels of β-carotene (Harjes et al. 2008). The reduction in *HYD3* transcripts leads to reduced conversion of β-carotene to downstream xanthophylls, causing β-carotene to accumulate (Vallabhaneni et al. 2009). Genetics tests such as the *HYD3* assay (Vallabhaneni et al. 2009) together with the previously described *LCYE* assay (Harjes et al. 2008) may be used to select germplasm containing optimal *HYD3* and *LCYE* alleles in breeding programs, which will lead to higher β-carotene levels in maize endosperm when both genes are highly expressed than either with optimal alleles of either gene alone. Furthermore, the experimental evidence from the association and linkage mapping reveals that *crtRB1* underlies a principal QTL associated with β-carotene concentration and conversion in maize kernels and *crtRB1* alleles associated with reduced transcript expressions correlate well with higher β-carotene (Yan et al. 2010). The most favorable *crtRB1* alleles that are rare in frequency and unique to temperate germplasm are being introgressed via inexpensive PCR-marker-assisted selection into tropical maize germplasm adapted to developing countries. A program at CIMMYT has already achieved

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Lpa1 locus (135 kb) fine mapped at RM3542 and RM482 and further delimited to a 47 kb region containing eight putative open reading frames	Andaya and Tai 2005
Soybean	
A locus on LG N near *Satt237* accounted for 41% variation in Pi, which is inversely related with Phyt, while another locus near *Satt237* on LG L explained 11% variation and interaction between the two loci accounted additional 8%–11% variation	Walker et al. 2006
Wheat	
Four QTL for P concentration and six QTL for P content; two QTL for Zn concentration on chromosomes 4A and 4D colocated with the QTLs for P concentration, while four QTL for Zn content on 2D, 3A, and 4A colocated with QTLs for P contents	Shi et al. 2008

which represents a key point in the metabolism of highly phosphorylated inositols, and could be a valuable tool in plant physiology to design low-phytate crops.

D. QTL Mapping, Cloning, and Introgression of β-Carotene into Adapted Germplasm

The natural plant pigments, termed as carotenoids, which are fat soluble, are important source of vitamin A and antioxidants. The two groups of carotenoids are provitamin A and nonprovitamin A carotenoids. The former includes three carotenoids, namely β-carotene, β-cryptoxanthin, and α-carotene, all precursor for vitamin A, while the latter consists of lutein and zeaxanthin, which act as antioxidants. Of all the carotenoids, β-carotene is the most widely distributed in plants and the one most efficiently converted to vitamin A. The consumption of carotenoid-rich foods is associated with reduced risks of developing cancer and cardiovascular diseases, enhanced immune responses, improved vision, and prevention of night blindness as well the maintenance of healthy skin and gastrointestinal or respiratory systems. Carotenoids in plants play a crucial role in photosynthesis, membrane stability, growth, and development (Menkir et al. 2008).

Unlike other agronomically important traits, only limited germplasm sets have been assessed for β-carotene, predominantly in chickpea, maize, pearl millet, sorghum, and wheat (Table 3.6), with some lines accumulating β-carotene as high as 7.6 $\mu g\, g^{-1}$ in pearl millet (Hash et al. 1997) and 15.6 $\mu g\, g^{-1}$ in maize (Harjes et al. 2008; Menkir et al. 2008). Genotypic differences for β-carotene have also been reported in rice: some germplasm had shown β-carotene in unpolished seeds while others having no β-carotene in unpolished seeds. The rice germplasm with β-carotene in unpolished seeds include Amarillo Cuba, Dudemasino, Sirendah Kuning, Bongkitan, Calibo, Khao Dawk Mali 105, and Klemas (Tan et al. 2005). Furthermore, Kandlakunta et al. (2008) in a comprehensive study involving major cereals and legumes and commonly consumed vegetables detected high β-carotene in chickpea (15.7 $\mu g\, g^{-1}$), green gram (12.8 $\mu g\, g^{-1}$), red gram (12.4 $\mu g\, g^{-1}$), and maize (17.1 $\mu g\, g^{-1}$), while among vegetables, they detected high β-carotene in yellow pumpkin (118 $\mu g\, g^{-1}$), green chillies (102 $\mu g\, g^{-1}$), field beans (55.4 $\mu g\, g^{-1}$), French bean (39.3 $\mu g\, g^{-1}$), ridge gourd (32.4 $\mu g\, g^{-1}$), green beans (23.9 $\mu g\, g^{-1}$), and brinjal (16.9 $\mu g\, g^{-1}$).

Maize is an important source of provitamin A (β-carotene, α-carotene, and β-cryptoxanthin) and the nonprovitamin A including lutein and zeaxanthin. Most of the yellow maize grains consumed worldwide have

QTL (Tiwari et al. 2010). The profiling of introgression using simple sequence repeats (SSRs), genomic *in situ* hybridization (GISH), and fluorescent *in situ* hybridization (FISH) analysis further confirmed the introgression of chromosome 2S, 2U, 7S, and 7U into these progenies (Singh et al. 2010a). More recently, genetic mapping identified five putative QTL for seed-Fe density and two QTL for seed-Zn density in pearl millet (Kumar et al. 2010b).

A number of QTL for seed phosphorus (P) and/or phytate concentrations have been reported in common bean, rice, sorghum, soybean, and wheat (Table 3.5), some with either major effects or colocated with QTL affecting seed-Fe or -Zn concentration. For example, a major QTL for P (19% variation) on b01 colocated with QTL accounting 34% variation each for seed Fe and Zn in common bean (Cichy et al. 2009). Furthermore, QTL for seed P or phytate concentration or content related to seed weight QTL on LGs b06, b07, and b10 (Blair et al. 2009c) or genes coding for candidate enzymes involved in phytic acid synthesis pathway and markers associated with each gene (Fileppi et al. 2010) have been mapped in common bean. In rice, the candidate gene for low-phytate mutant alleles and markers (LPA1_CAPS for *lpa1-1* and LPA1_InDel for *lpa1-2*) showed complete cosegregation with mutant phenotypes (Zhao et al. 2008). Furthermore, the two QTL for seed-Zn concentration on chromosomes 4A and 4D colocated with QTL for P concentration, while four QTL for seed-Zn content on chromosome 2D, 3A, and 4A colocated with the QTL for P content, reflecting positive correlation between the seed-Zn and -P concentrations (see Section VI.B), which may provide opportunities for simultaneous improvement in seed-P and -Zn density in wheat (Shi et al. 2008).

Two QTL mapped onto LGs L and N control low phytate in soybean line, CX 1834 (Walker et al. 2006; Gao et al. 2008). Further, Saghai-Maroof et al. (2009) mapped and sequenced a putative multidrug resistance-associated protein (MRP) gene on LG N that contributes to low-phytate phenotype in CX 1834. This A to T mutation provided a single nucleotide polymorphism (SNP) marker for introgressing the low-phytate QTL from CX 1834 into desired breeding lines (Saghai-Maroof et al. 2009).

More recently, genes coding for candidate enzymes involved in the phytic acid pathway have been mapped and identified markers associated with each gene (*PvMIPSs*, *PvMIPSv*, *PvIMP*, *PvMIK*, *PvIPK2*, *PvITPKα*, *PvITPKβ*, *PvIPK1*), which may represent a useful resource to select genetic variants with low-phytate trait in common bean (Fileppi et al. 2010). Furthermore, González et al. (2010) discovered how phytate is produced in plants, by solving the structure of the protein $InsP_5$ 2-Kinase (IP_5 2-K), a distant member of the IPK family,

Table 3.5. Summary of marker/QTL analysis of seed phosphorus (P) and phytate (Phyt) concentration in barley, common bean, rice, sorghum, soybean, and wheat covering a period from 2005 to 2010.

QTL summary	References
Barley	
Flanking markers mapped 3 low phytic acid mutant alleles: *lpa1* at EBmac415 and Msu21; *lpa2-1* at Bmag120 and AWBMS0022; *lpa678* at EBmac701 and Bmag714B	Oliver et al. 2009
Common bean	
Genes coding for candidate enzymes involved in phytic acid pathway mapped and markers associated with each gene (*PvMIPSs*, *PvMIPSv*, *PvIMP*, *PvMIK*, *PvIPK2*, *PvITPKά*, *PvITPKβ*, *PvIPK1*) identified	Fileppi et al. 2010
Eight QTL for P on six LGs, in an Andean × Andean bush bean cross, 11%–40% variation; two QTL for Phyt on two LGs, 17%–18% variation; a QTL for P (19% variation) on B1 colocated with QTLs accounting 34% variation each for Fe and Zn on same LG	Cichy et al. 2009
Six QTL for P on LG B2 and B6 and three QTL for Phyt on LG B6, with QTL for P or Phyt were related to seed weight QTL on LGs B6, B7, and B10 in an inter-gene pool mapping population	Blair et al. 2009b
Rice	
Mutant alleles of the low phyt homozygous lethal (*XS-lpa2-1*) and nonlethal (*XS-lpa-2-2*) mapped: *XS-lpa2-1* gene to a region on chromosome 3 between marker RM14360 and RM1332, where also located the rice orthologue (*OsMRP5*) of the maize *lpa1*; a single base pair change in the sixth exon of *XS-lpa2-1* and a 5-bp deletion in the first exon of *XS-lpa2-2* resulted these mutations	Xu et al. 2009
TIGR locus LOC_Os02g57400 identified as the candidate gene for mutant alleles: *lpa1-1* is a single base pair substitution mutation while *lpa1-2* involves a 1,475 bp fragment deletion; a CAPS marker (LPA1_CAPS) for *lpa1-1* and an InDel marker (LPA1_InDel) for *lpa1-2* confirmed complete segregation with LPA phenotypes	Zhao et al. 2008
A single base pair change resulted *lpa* mutant, N15-186, mapped at RM15875 and RM15907 on chromosome 3, which also harbor rice orthologue of maize *lpa3*; *lpa* N15-186 is a mutant allele of the rice *myo*-inositol kinase (*Os MIK*) gene	Kim et al. 2008

(*continued*)

Table 3.4 (*Continued*)

QTL summary	References
Rice	
Two QTL for Fe on chromosome 2 and 9 and three QTL for Zn on chromosome 5, 8, and 12; a major QTL for Zn on chromosome 8 accounted 11%–19% variation	Garcia-Oliveira et al. 2009
Three QTL for Fe on chromosomes 2, 8, and 12, while two QTL for Zn on 1 and 12; a common QTL for Fe and Zn on chromosome 12 accounted 13%–14% variation	Stangoulis et al. 2007
Wheat	
Two QTL for seed Fe on chromosomes 2A and 7A, *QFe.pau-2A* and *QFe.pau-7A* explaining 12%–13% variation, and one QTL for seed Zn on chromosome 7A, QZn.pau-7A explaining 19% variation	Singh et al. 2010a
Two QTLs for Fe on chromosomes 2A and 7A, mapped at *Xwmc382–Xbarc124* and *Xgwm473–Xbarc29*, explained 12%–13% variation; a QTL for Zn on chromosome 7A, mapped at *Xcfd31–Xcfa2049*, explained 19% variation	Tiwari et al. 2009
A QTL on chromosome 3D for seed-Fe and four QTLs on chromosomes 3D, 4B, 6B, and 7A for Zn concentrations; QTL for seed-Fe concentration colocated with a QTL for shoot-Fe concentration and seed weight, with alleles for high Fe concentration coming from the same parent	Genc et al. 2009
11 QTLs on chromosomes 2A, 5A, 6B, 7A, and 7B for Fe and 6 QTL on chromosomes 2A, 2B, 3A, 4B, 5A, 6A, 6B, 7A, and 7B for Zn; clusters of QTLs on chromosome 2A, 5A, 6B, and 7A for seed protein and minerals	Peleg et al. 2009
Four QTLs for Zn concentration and seven QTLs for Zn content, with concentration QTLs colocated with those of content QTLs, possible to improve both traits simultaneously	Shi et al. 2008

phenotypic variation (Stangoulis et al. 2007). In barley, specific markers have been developed for a high zinc QTL on chromosome 2H (Sadeghzadeh et al. 2010), while other QTLs have been identified by Lonergan et al. (2009).

Iron reductases are members of the protein super-family of flavocytochromes and function in roots to convert Fe from a plant unavailable form (ferric, Fe^{3+}) to an available form (ferrous, Fe^{2+}) that can be readily absorbed (Grusak 1995). IRA is known to vary with plant growth conditions (e.g., soil pH and available iron concentration) (Grusak 2000). Common bean genotypes with high seed-Fe showed high IRA than those with low seed-Fe, suggesting a link between root uptake and seed loading of Fe in common bean (Grusak 1994, 2000, 2002). More recently, Blair et al. (2010b) reported a single major QTL for IRA under Fe-limited conditions (1 μM) on LG b02, and another major QTL under Fe-sufficient conditions (15 μM) on LG b11 that was associated with several QTL for seed Fe in common bean. Thus, the QTL for IRA under Fe-limited conditions may be useful in environments where beans are grown in alkaline soils, while the QTL for IRA under Fe-sufficient conditions may be useful for selecting for enhanced seed nutritional quality (Blair et al. 2010b).

Wild emmer wheat germplasm harbors a rich allelic diversity, including for seed minerals (Xie and Nevo 2008). A major locus, *Gpc-B1* (a 250-kb locus) mapped as a simple Mendelian locus (Distelfeld et al. 2006), associated with increased seed-protein (38%), -Fe (18%), and -Zn (12%) concentrations from wild emmer wheat germplasm (*Triticum dicoccoides*), encodes a NAC transcription factor (*NAM-B1*) that accelerates senescence and increases nutrient remobilization from leaves to developing seeds (Uauy et al. 2006; Distelfeld et al. 2007). *Triticum turgidum* is another useful wild emmer germplasm for improving seed mineral concentration in wheat. Peleg et al. (2009) mapped 82 QTL for 10 seed minerals (LOD score range of 3–17), with most of the positive alleles contributed by wild emmer accession, G18-16, and many QTL for the same trait mapped to homoeologous positions, reflecting synteny between the A and B genomes. *TtNAM-B1* affecting seed-protein, -Fe, and -Zn originating from wild emmer wheat has been cloned (Distelfeld and Fahima 2007). Furthermore, Singh et al. (2010a) identified two QTL (*QFe.pau-2A* and *QFe.pau-7A*) for Fe and a QTL (*QZn.pau-7A*) for Zn, which they transferred into interspecific progenies involving *Aegilops kotschyi* and *Aegilops peregrine*, both UUSS genome species. Such progenies showed 60%–136% enhanced seed-Fe and -Zn concentrations and 50%–120% increased Fe and Zn contents per seed as compared to the control cultivar that was introgressed with these

for desirable genes in crop breeding (Dwivedi et al. 2007; Collard and Mackill 2008). Automation of high-throughput assays including next generation sequencing technologies and associated data mining tools provide breeders/molecular biologists opportunities to handle and interpret large data sets (Varshney et al. 2009; Feuillet et al. 2010). Furthermore, the genomes of agriculturally important crops such as maize, rice, sorghum, and soybean have been sequenced (IRGSP 2005; Paterson et al. 2009; Schanable et al. 2009; Schmutz et al. 2010), while several projects under way to sequence genomes of many other agriculturally important food crops (Feuillet et al. 2010). The deoxyribonucleic acid (DNA) sequence variants across species or among strains within a species may be used as new genetic tools for developing markers and subsequently crop cultivars with specific characteristics.

The crops included in this review have abundant genetic resources to dissect population structure and diversity in germplasm collections to identify genetically diverse germplasm with beneficial traits. However, only recently have DNA marker-based technologies been used to identify QTL associated with increased seed-Fe and -Zn concentrations in barley, common bean, pearl millet, rice, and wheat, revealing many QTL with varying effects; some with major phenotypic variation while many others with minor effects (Table 3.4). For example, in common bean a QTL on linkage group (LG) b09 was found for Zn by Gelin et al. (2007), while QTL found by Cichy et al. (2009) on LG b01 (near the *fin* gene) accounted for 34% of variation for seed-Fe and -Zn concentrations and also overlapped with a major QTL (19% variation) for increased seed phosphorus (P) concentration. Finally, other QTL on LG b06 accounted for 36% variation for seed Fe and 39% variation for seed Zn that same study (Cichy et al. 2009). This latter QTL was linked with a QTL found for Mesoamerican beans by Blair et al. (2010c). Further studies by Blair et al. (2009b, 2010b) in both inter- and intragene pool populations, respectively, found specific major and minor QTL for Fe and Zn concentrations with the former type mainly on LG b11. Therefore, at least four major QTL have been identified in common bean affecting micronutrient concentration depending on the gene pool and genetic background of the material tested.

In other crops, meanwhile, a QTL for seed-Zn on chromosome 7A mapped at *Xcfd31–Xcfa2049* explained 19% variation in wheat (Tiwari et al. 2009). In rice, a seed-Zn QTL mapped at *RM235–RM17* on chromosome 12 accounted for 13% variation and colocated with seed-Fe QTL that mapped at *RM270–RM17* and accounted 14%

Table 3.4. Summary of marker/QTL analysis of seed iron (Fe) and/or zinc (Zn) concentration in barley, common bean, rice, and wheat covering a period from 2003 to 2011.

QTL summary	References
Barley	
Sequence-specific PCR-based dominant marker, SZnR1, located on the short arm of chromosome 2H, associated with high seed zinc concentration and content	Sadeghzadeh et al. 2010
Three of the five most favorable QTL alleles increased seed-Zn concentration and content by an average of 53% and 75%, respectively	Lonergan et al. 2009
Common bean	
Total of nine seed mineral QTL were identified in an Andean × Andean mapping population on five linkage groups (LGs) with the most important being new loci on b02 and other QTL on b06, b08, and b07 near phaseolin. Seed weight QTL were associated with these on b02 and b08	Blair et al. 2011
A set of across site, overlapping iron and zinc QTL were discovered for a Mesoamerican × Mesoamerican mapping population on LG b06 suggesting a possibly pleiotropic locus and common physiology for mineral uptake or loading. Other QTL for mineral concentration or content were found on LGs b02, b03, b04, b07, b08, and b11 and together with the b06 cluster were mostly novel compared to loci found in previous studies of the Andean gene pool or inter-gene pool crosses	Blair et al. 2010c
Total of 26 QTL were identified in an inter-gene pool mapping population for the mineral × trial × method combinations of which half were for iron concentration and half for zinc concentration. Many of the QTL (11) for both iron (5) and zinc (6) clustered on the upper half of LG B11, explaining up to 47.9% of phenotypic variance, suggesting an important locus useful for marker-assisted selection. Other QTL were identified on LG B3, B6, B7, and B9 for zinc and B4, B6, B7, and B8 for iron	Blair et al. 2009a
Fe: 11 QTLs on six LGs, 8%–36% variation; Zn: 11 QTL on four LGs, 9%–39% variation; a QTL on LG B1 (nearest to *fin* marker) accounted 34% variation for Fe and Zn, while another QTL on LG B6 (nearest to *AGAT05* marker) accounted 36%–39% variation for Fe and Zn	Cichy et al. 2009
A locus on LG9 associated with Zn accumulation	Gelin et al. 2007
Two QTL for Fe (25% variation) and one QTL for Zn (15% variation)	Guzmán-Maldonado et al. 2003

(*continued*)

Table 3.3 (*Continued*)

Number and type of experimental material	Correlation coefficient	References
Sorghum		
29 Core collection accessions	0.75**	Kumar et al. 2009
84 Including hybrid parental lines, germplasm, cultivars, and those differing in seed quality traits	0.55**	Reddy et al. 2005
Wheat		
600 Core collection accessions of diverse origin	0.81***	Velu et al. 2011
154 Genotypes including wild species and 2 environments	0.40***–0.67***	Chatzav et al. 2010
265 Lines including leading cultivars and advanced lines	0.75***	Zhang et al. 2010
152 RILs involving durum and wild emmer wheat, 3 environments	0.79***	Peleg et al. 2009
90 Double haploid lines, tested at 2 locations for 2 years	0.55*–0.84*	Genc et al. 2009
175 Diverse lines involving bread (winter/spring), durum, spelt, einkorn, and emmer wheat's	0.29**	Zhao et al. 2009
19 Wild emmer germplasm, and 5 environments	0.50**	Gómez-Becerra et al. 2010
66 Winter/spring wheat cultivars and improved lines and 5 locations	0.79***	Morgounov et al. 2007

Abbreviations: RIL = recombinant inbred line population individuals.

Highly significant and positive correlations (0.82–0.99) between seed P and phytate have been reported for common bean, pearl millet, and rice (Lolas and Markakis 1975; Stangoulis et al. 2007; Selvi and Rajarathinam 2009), while a low but positive and significant association was found for seed phytate with Fe and Zn in common bean (Cichy et al. 2009). Further, several studies in wheat and common bean revealed a moderate but positive and significant association of seed P with Fe (0.42–0.55) and Zn (0.46–0.63) (Gelin et al. 2007; Peleg et al. 2009; Zhao et al. 2009; Zhang et al. 2010). The implications of the above are that while it should be possible to breed for high seed-Fe and -Zn concentrations with reduced phytate concentration in pearl millet, this may not be possible in wheat or common bean.

In yet another issue of correlations and micronutrient versus macronutrient concentrations, seed yield is significantly and positively associated with seed weight in cereals and legumes (Upadhyaya et al. 2002; García del Moral et al. 2003; Upadhyaya 2003; Maman et al. 2004; Morgounov et al. 2007). However, it is either not associated with seed-Fe and -Zn concentrations in pearl millet or shows a low but significant negative association in wheat (Morgounov et al. 2007; Peleg et al. 2009; Zhao et al. 2009) and positive association in common bean (Gelin et al. 2007). A negative association may pose problems for breeding of seed mineral-dense cultivars with high seed yield per se. Seed weight in pearl millet is highly significant and positively associated with seed Fe ($r = 0.80$) and Zn ($r = 0.85$) (Velu et al. 2007), while it is significant and positively correlated ($r = 0.61$) with Fe in common bean (Gelin et al. 2007).

Another question is whether there are any relationships between seed-Fe (or -Zn) concentration and bioavailability. Limited studies on seed-Fe and -Zn concentration and bioavailability in maize, rice, and wheat revealed no such associations (Glahn et al. 2002; Oikeh et al. 2003a,b,2004a), indicating that it is possible to significantly increase both concentration and bioavailability of either Fe or Zn in the seed by breeding and selection.

C. Quantitative Trait Loci (QTL) Associated with Seed Iron, Zinc, and Phytate Concentrations

Genomics science since the 1990s has made phenomenal advances toward developing a large number of molecular markers and genetic linkage maps allowing the mapping and/or cloning of QTL and identification of candidate gene(s) associated with agriculturally beneficial traits, which can lead marker-assisted selection (MAS)

gradually from the aleurone/pericarp and outer parts of the endosperm to the interior of the endosperm, while Mn and Fe very much localize in the aleurone/pericarp region with a sharp change in the concentration in the exterior parts of the endosperm. Mn is highly concentrated in the embryo but with a different pattern than observed for Zn. The strong similarities between the distribution of Fe, Mn, and P and between Zn and S may be indicative of the complexation mechanisms involved in rice seeds. Preliminary studies in pearl millet revealed greater concentration of seed minerals including Fe and Zn in the covering layers and the germ than in the endosperm portions, similar to most cereal seeds (Varriano-Marston and Hoseney 1980).

Common bean and soybean genotypes were reported to accumulate different proportion of total seed Fe in the seed coat, embryo, and cotyledons (Laszlo 1990; Moraghan and Grafton 2002; Moraghan et al. 2002; Moraghan 2004; Ariza-Nieto et al. 2007; Cvitanich et al. 2010, 2011), indicating that specific tissues relevant for Fe storage should be identified and their Fe loading mechanisms be investigated to exploit such variability toward developing seed iron-dense cultivars. Using PIXE assay to investigate Fe distribution in seed tissues of *Phaseolus* species, Cvitanich et al. (2010, 2011) concluded that (1) the distribution of Fe in seed depends on the species and genotype, (2) high concentrations of Fe accumulate in cells surrounding the provascular tissue, (3) the tissue in the proximity of the provascular bundles holds up to 500 $\mu g\, g^{-1}$ Fe, depending on genotypes, and (4) the largest proportion of seed Fe in *Phaseolus* species is stored in compounds and cell parts different from ferritin and starch vacuoles. These results indicate that more studies are needed to assess the patterns of micronutrient distribution in seeds, and that micronutrient distribution criteria should be integrated into the selection strategies for biofortification of staple crops.

In summary, CGIAR and national agricultural research systems (NARS) institutions hold large collection of germplasm, both cultivated and wild relatives' of cereal and legume crops. The core or mini-core collections available in these crops may be used to identify seed mineral-dense germplasm. The germplasm from regions deficient in soil micronutrients should receive priority for evaluation as such germplasm are expected to develop inherent adaptation mechanisms that favor enhanced nutrient acquisition, transport, distribution, and relocation in plants/seeds. Few germplasm lines with high seed-Fe and/or -Zn concentrations have been reported in common bean, maize, pearl millet, rice, sorghum, and wheat. Wild and weedy relatives of common bean and wheat have shown abundant variability for Fe and Zn. Mutants with moderate-to-high reduction in phytate are available in

barley, common bean, maize, rice, soybean, and wheat. Genotypic differences in iron bioavailability have been reported in common bean, maize, rice, and wheat, which should be further explored. Several methods are available with high precision to map elements distribution, which may be used to identify barriers to Fe and Zn accumulation in the seed.

VI. EXPLOITING NATURAL GENETIC VARIATION TO BREED FOR SEED MINERAL-DENSE CULTIVARS

A. Fixing the Biologically Attainable Target to Breed for Seed Mineral-Dense Crops

Several factors must be taken into consideration when setting the target levels for enhancing the nutritional status of food crops by breeding. These include (1) mapping the human populations with micronutrient deficiency, (2) food habits of those suffering from micronutrient malnutrition, (3) the major staple crops grown in micronutrient-deficient regions and their nutrient profiles, (4) the recommended micronutrient requirement vis-à-vis daily nutrient intake, (5) the genetic variation for micronutrients in germplasm pools and cultivars/hybrids produced in the region or of possible production there, and (6) the bioavailability, bioconversion, and bioaccessibility of the micronutrients in the crop or combination of crops consumed in the diet (Nestel et al. 2006; Ortiz-Monasterio et al. 2007; Pfeiffer and McClafferty 2007; Bouis and Welch 2010). The target set for crop biofortification for one nutrient may not be the same for a different micronutrient, and may further differ from one country or region to another. The baseline data of daily intake of minerals may vary as detected for iron concentration in India. The intake of iron in India is less than 50% of the recommended dietary allowance, and iron density is about 8.5 mg 1000 $Kcal^{-1}$, with significant differences in absolute amounts among regions. Diets in Indian state of Andhra Pradesh with rice as staple have lowest iron (7 mg 1000 $Kcal^{-1}$), while diets in Gujarat and Madhya Pradesh with pearl millet as the staple have the highest iron intake (16 mg 1000 $Kcal^{-1}$) (Nair and Iyengar 2009). Likewise, several target regions for crop biofortification (Fe, Zn, and provitamin A) interventions have been identified in Latin America and the Caribbean (Zapata-Caldas et al. 2009). For example, interventions in northern Colombia appear promising for all crops, while sites for bean biofortification are widely scattered throughout the country. The most promising sites in Nicaragua are found in the

center-north region, while candidate sites for biofortification in Bolivia are found in the central part of the country and in the Andes Mountains. Poverty levels indicated that northeast Brazil is the most important region for biofortification in that part of South America.

Variations in the conversion factor of provitamin A (β-carotene) to vitamin A (retinol) in food crops should also need to be considered when defining breeding targets for β-carotene (Tang 2010). Significant genetic variation for seed-Fe and -Zn concentrations has been reported for cereal and legume crops, with some genotypes having more bioavailable seed micronutrients than others (see Section V.A). Most modern cultivars/hybrids have lower micronutrients per se than those reported in germplasm pools of a given species (Graham et al. 1999; Frossard et al. 2000). The adverse effects of processing, storage, and cooking on nutrient concentrations losses are known (see Section III.B.2). In addition, there are certain elements present in the seed that either act as enhancers (i.e., ascorbic acid) or inhibitors (i.e., phytase) of micronutrient uptake and absorption (see Section III.B.1).

All these variables need to be factored in when setting the breeding targets for improved nutritional quality of food crops. HarvestPlus has set the tentative breeding targets for improving the micronutrient density of several food crops. For example, the tentative targets to increase seed-Fe concentration of rice, wheat, pearl millet, common beans, maize, cassava, and sweet potato are 15, 59, 88, 107, 60, 45, and 85 $\mu g\, g^{-1}$ on a dry weight basis, respectively, while those for Zn are fixed at 28, 38, 66, 56, 38, 34, and 70 $\mu g\, g^{-1}$ (Bouis and Welch 2010).

For provitamin A, the targets set are 17, 17, 23, 34, 17, 48, and 91 $\mu g\, g^{-1}$ for rice, wheat, pearl millet, common bean, maize, cassava, and sweet potato, respectively (Bouis and Welch 2010). Such an approach can be applied to define the target levels for other micronutrients as well. However, targets should be dynamic depending on the severity of the micronutrient deficiency and the progress realized through breeding for developing mineral-dense cultivars/hybrids.

B. Genotype × Environment Interaction and Relationships Between Seed Minerals and Agronomic Traits

Knowledge of the effects of G, environments (E), and genotype × environment interaction (GEI) is important for developing nutritionally enhanced crop cultivars. Like yield and yield attributing traits, seed-Fe and -Zn concentrations in common bean, maize, rice, and wheat are influenced by location (or E), G, and GEI (Table 3.2), with location effects generally much larger than those of either G or GEI effects. The

Table 3.2. Environment (location) and genotype (G) × environment (E) interaction effects on seed-iron (Fe) and -zinc (Zn) content in common bean, maize, rice, and wheat covering a period from 2003 to 2010.

Fe			Zn			
Genotype	Environment	G × E interaction	Genotype	Environment	G × E interaction	References
Common bean						
***	***	*	***	***	NS	Astudillo and Blair 2008
Maize						
* (15%)	** (31%)	* (28%)	** (15%)	*** (26%)	** (35%)	Oikeh et al. 2004b
	NS	**		NS	**	Oikeh et al. 2003b
Rice						
	*	NS				Abilgos-Ramos et al. 2004
Wheat						
*** (9%)	*** (71%)	*** (20%)	*** (19%)	*** (65%)	*** (16%)	Gómez-Becerra et al. 2010
*** (10%)	*** (86%)	*** (3%)	*** (3%)	*** (89%)	*** (8%)	Zhang et al. 2010
NS	** (99%)	NS	NS	** (92%)	NS	Joshi et al. 2010
*** (31%)	*** (9%)	*** (54%)	*** (34%)	*** (4%)	*** (51%)	Ficco et al. 2009
* (50%)	** (10%–51%)	(18%–48%)	* (9%)	** (32%–84%)	(7%–49%)	Morgounov et al. 2007
* (1%)	*** (98%)	NS	* (1%)	*** (98%)	NS	Distelfeld et al. 2007

Notes: *, **, and *** indicate significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Figures in the parenthesis refer to % phenotypic variation. Ns, nonsignificant

growing environments had no effect on bioavailable Fe in maize (Oikeh et al. 2004a); however, Pixley et al. (2011a) detected larger E than GEI for Fe bioavailability in maize. It is therefore suggested that responses of cultivars to different production environments need to be well understood to improve the probability of predicting and identifying cultivars that are not only high in seed-Fe and/or -Zn concentrations but also these micronutrients are more bioavailable to absorption (Briat and Lobreaux 1997; Pixley et al. 2011a).

The environmental variables such as pH, temperature, solar radiation, precipitation, organic matter, and soil texture have the potential to influence nutrient concentration (Tisdale and Nelson 1975; Römheld and Marschner 1986; Cabuslay et al. 2003; Abilgos-Ramos et al. 2004; Joshi et al. 2010) and must be taken into consideration while explaining the variation for seed micronutrients in germplasm or when assessing the nutritional quality of staple food crops grown in diverse agroecological conditions.

Character association between seed mineral concentrations may indicate the existence of one or more common genetic-physiological mechanisms involved in mineral uptake by the root system, translocation, and redistribution within the plant tissues, remobilization to the seed, and accumulation in the developing seed (Chatzav et al. 2010). Both mineral concentration (amount per unit weight, i.e., $mg\ kg^{-1}$) and mineral content (amount per seed, i.e., $\mu g\ seed^{-1}$) are positively correlated (Cakmak et al. 2004; Hacisalihoglu et al. 2005; Stangoulis et al. 2007) and either can be used to estimate the quantity of the minerals in the seeds (see Section V.A).

An understanding of the nature of association between different minerals and also with seed yield and seed weight (100 or 1,000-seed weight) should facilitate the selection of mineral-dense progenies in breeding. The published evidence suggests that seed-Fe and -Zn concentrations, in most cases, are highly significant and positively correlated in common bean, pearl millet, rice, and wheat (Table 3.3), which suggests that genes for Fe and Zn accumulation cosegregate or are pleiotropic. Such relationships could be exploited toward selecting progenies with high seed minerals in the segregating populations. Further studies revealed that Fe and Zn in the flag leaves of *Aegilops* species are highly significant and positively correlated with seed Fe and Zn (Rawat et al. 2009a,b); however, such relationships were not found in common bean (Tryphone and Nchimbi-Msolla 2010). More studies are needed to elucidate these relationships prior to using flag leaf for early selection of plants with potentially high seed Fe and Zn in breeding programs.

Table 3.3. Relationships among seed-iron (Fe) and -zinc (Zn) contents in common bean, maize, pearl millet, rice, sorghum, and wheat covering a period from 2003 to 2010.

Number and type of experimental material	Correlation coefficient	References
Common bean		
90 Genotypes from Tanzania	0.416**	Tryphone and Nchimbi-Msolla 2010
110 RILs involving G14519 and G4825, three sites	0.483**, 0.636***, 0.686***	Blair et al. 2010a
87 RIL involving G19833 and DOR364, two sites, two methods	0.594**–0.751**	Blair et al. 2009b
10 Cultivars	0.75***	Oomah et al. 2008
40 Colombian varieties, low and high P soils	0.59**, 0.75***	Astudillo and Blair 2008
76 RILs involving Jamapa and Calima	0.60**	Hacisalihoglu et al. 2005
24 Germplasm accessions	0.65*	Welch et al. 2000a
Maize		
294 F_4 lines and six controls including parental lines; two environments	0.13**–0.14**	Šimic et al. 2009
21 Late-maturing varieties and 3 locations	0.48***	Oikeh et al. 2004a
49 Late-maturing tropical maize and 3 environments	0.51***	Oikeh et al. 2003
Pearl millet		
61 Genotypes	0.88**	Selvi and Rajarathinam 2009
54 S_1 progenies from two populations	0.80**–0.82**	Gupta et al. 2009
120 Lines including populations and population progenies, germplasm, seed parents, and pollinators	0.84**	Velu et al. 2007
79 Lines including male steriles, testers, and hybrids	0.56**	Arulselvi et al. 2007
Rice		
202 Traditional and modern cultivars	0.346**	Anandan et al. 2011
129 Double haploid lines involving IR64 and Azucena	0.71***	Stangoulis et al. 2007

(*continued*)

Table 3.3 (*Continued*)

Number and type of experimental material	Correlation coefficient	References
Sorghum		
29 Core collection accessions	0.75**	Kumar et al. 2009
84 Including hybrid parental lines, germplasm, cultivars, and those differing in seed quality traits	0.55**	Reddy et al. 2005
Wheat		
600 Core collection accessions of diverse origin	0.81***	Velu et al. 2011
154 Genotypes including wild species and 2 environments	0.40***–0.67***	Chatzav et al. 2010
265 Lines including leading cultivars and advanced lines	0.75***	Zhang et al. 2010
152 RILs involving durum and wild emmer wheat, 3 environments	0.79***	Peleg et al. 2009
90 Double haploid lines, tested at 2 locations for 2 years	0.55*–0.84*	Genc et al. 2009
175 Diverse lines involving bread (winter/spring), durum, spelt, einkorn, and emmer wheat's	0.20**	Zhao et al. 2009
19 Wild emmer germplasm, and 5 environments	0.50**	Gómez-Becerra et al. 2010
66 Winter/spring wheat cultivars and improved lines and 5 locations	0.79***	Morgounov et al. 2007

Abbreviations: RIL = recombinant inbred line population individuals.

Highly significant and positive correlations (0.82–0.99) between seed P and phytate have been reported for common bean, pearl millet, and rice (Lolas and Markakis 1975; Stangoulis et al. 2007; Selvi and Rajarathinam 2009), while a low but positive and significant association was found for seed phytate with Fe and Zn in common bean (Cichy et al. 2009). Further, several studies in wheat and common bean revealed a moderate but positive and significant association of seed P with Fe (0.42–0.55) and Zn (0.46–0.63) (Gelin et al. 2007; Peleg et al. 2009; Zhao et al. 2009; Zhang et al. 2010). The implications of the above are that while it should be possible to breed for high seed-Fe and -Zn concentrations with reduced phytate concentration in pearl millet, this may not be possible in wheat or common bean.

In yet another issue of correlations and micronutrient versus macronutrient concentrations, seed yield is significantly and positively associated with seed weight in cereals and legumes (Upadhyaya et al. 2002; García del Moral et al. 2003; Upadhyaya 2003; Maman et al. 2004; Morgounov et al. 2007). However, it is either not associated with seed-Fe and -Zn concentrations in pearl millet or shows a low but significant negative association in wheat (Morgounov et al. 2007; Peleg et al. 2009; Zhao et al. 2009) and positive association in common bean (Gelin et al. 2007). A negative association may pose problems for breeding of seed mineral-dense cultivars with high seed yield per se. Seed weight in pearl millet is highly significant and positively associated with seed Fe ($r = 0.80$) and Zn ($r = 0.85$) (Velu et al. 2007), while it is significant and positively correlated ($r = 0.61$) with Fe in common bean (Gelin et al. 2007).

Another question is whether there are any relationships between seed-Fe (or -Zn) concentration and bioavailability. Limited studies on seed-Fe and -Zn concentration and bioavailability in maize, rice, and wheat revealed no such associations (Glahn et al. 2002; Oikeh et al. 2003a,b,2004a), indicating that it is possible to significantly increase both concentration and bioavailability of either Fe or Zn in the seed by breeding and selection.

C. Quantitative Trait Loci (QTL) Associated with Seed Iron, Zinc, and Phytate Concentrations

Genomics science since the 1990s has made phenomenal advances toward developing a large number of molecular markers and genetic linkage maps allowing the mapping and/or cloning of QTL and identification of candidate gene(s) associated with agriculturally beneficial traits, which can lead marker-assisted selection (MAS)

for desirable genes in crop breeding (Dwivedi et al. 2007; Collard and Mackill 2008). Automation of high-throughput assays including next generation sequencing technologies and associated data mining tools provide breeders/molecular biologists opportunities to handle and interpret large data sets (Varshney et al. 2009; Feuillet et al. 2010). Furthermore, the genomes of agriculturally important crops such as maize, rice, sorghum, and soybean have been sequenced (IRGSP 2005; Paterson et al. 2009; Schanable et al. 2009; Schmutz et al. 2010), while several projects under way to sequence genomes of many other agriculturally important food crops (Feuillet et al. 2010). The deoxyribonucleic acid (DNA) sequence variants across species or among strains within a species may be used as new genetic tools for developing markers and subsequently crop cultivars with specific characteristics.

The crops included in this review have abundant genetic resources to dissect population structure and diversity in germplasm collections to identify genetically diverse germplasm with beneficial traits. However, only recently have DNA marker-based technologies been used to identify QTL associated with increased seed-Fe and -Zn concentrations in barley, common bean, pearl millet, rice, and wheat, revealing many QTL with varying effects; some with major phenotypic variation while many others with minor effects (Table 3.4). For example, in common bean a QTL on linkage group (LG) b09 was found for Zn by Gelin et al. (2007), while QTL found by Cichy et al. (2009) on LG b01 (near the *fin* gene) accounted for 34% of variation for seed-Fe and -Zn concentrations and also overlapped with a major QTL (19% variation) for increased seed phosphorus (P) concentration. Finally, other QTL on LG b06 accounted for 36% variation for seed Fe and 39% variation for seed Zn that same study (Cichy et al. 2009). This latter QTL was linked with a QTL found for Mesoamerican beans by Blair et al. (2010c). Further studies by Blair et al. (2009b, 2010b) in both inter- and intragene pool populations, respectively, found specific major and minor QTL for Fe and Zn concentrations with the former type mainly on LG b11. Therefore, at least four major QTL have been identified in common bean affecting micronutrient concentration depending on the gene pool and genetic background of the material tested.

In other crops, meanwhile, a QTL for seed-Zn on chromosome 7A mapped at *Xcfd31–Xcfa2049* explained 19% variation in wheat (Tiwari et al. 2009). In rice, a seed-Zn QTL mapped at *RM235–RM17* on chromosome 12 accounted for 13% variation and colocated with seed-Fe QTL that mapped at *RM270–RM17* and accounted 14%

Table 3.4. Summary of marker/QTL analysis of seed iron (Fe) and/or zinc (Zn) concentration in barley, common bean, rice, and wheat covering a period from 2003 to 2011.

QTL summary	References
Barley	
Sequence-specific PCR-based dominant marker, SZnR1, located on the short arm of chromosome 2H, associated with high seed zinc concentration and content	Sadeghzadeh et al. 2010
Three of the five most favorable QTL alleles increased seed-Zn concentration and content by an average of 53% and 75%, respectively	Lonergan et al. 2009
Common bean	
Total of nine seed mineral QTL were identified in an Andean × Andean mapping population on five linkage groups (LGs) with the most important being new loci on b02 and other QTL on b06, b08, and b07 near phaseolin. Seed weight QTL were associated with these on b02 and b08	Blair et al. 2011
A set of across site, overlapping iron and zinc QTL were discovered for a Mesoamerican × Mesoamerican mapping population on LG b06 suggesting a possibly pleiotropic locus and common physiology for mineral uptake or loading. Other QTL for mineral concentration or content were found on LGs b02, b03, b04, b07, b08, and b11 and together with the b06 cluster were mostly novel compared to loci found in previous studies of the Andean gene pool or inter-gene pool crosses	Blair et al. 2010c
Total of 26 QTL were identified in an inter-gene pool mapping population for the mineral × trial × method combinations of which half were for iron concentration and half for zinc concentration. Many of the QTL (11) for both iron (5) and zinc (6) clustered on the upper half of LG B11, explaining up to 47.9% of phenotypic variance, suggesting an important locus useful for marker-assisted selection. Other QTL were identified on LG B3, B6, B7, and B9 for zinc and B4, B6, B7, and B8 for iron	Blair et al. 2009a
Fe: 11 QTLs on six LGs, 8%–36% variation; Zn: 11 QTL on four LGs, 9%–39% variation; a QTL on LG B1 (nearest to *fin* marker) accounted 34% variation for Fe and Zn, while another QTL on LG B6 (nearest to *AGAT05* marker) accounted 36%–39% variation for Fe and Zn	Cichy et al. 2009
A locus on LG9 associated with Zn accumulation	Gelin et al. 2007
Two QTL for Fe (25% variation) and one QTL for Zn (15% variation)	Guzmán-Maldonado et al. 2003

(*continued*)

Table 3.4 (*Continued*)

QTL summary	References
Rice	
Two QTL for Fe on chromosome 2 and 9 and three QTL for Zn on chromosome 5, 8, and 12; a major QTL for Zn on chromosome 8 accounted 11%–19% variation	Garcia-Oliveira et al. 2009
Three QTL for Fe on chromosomes 2, 8, and 12, while two QTL for Zn on 1 and 12; a common QTL for Fe and Zn on chromosome 12 accounted 13%–14% variation	Stangoulis et al. 2007
Wheat	
Two QTL for seed Fe on chromosomes 2A and 7A, *QFe.pau-2A* and *QFe.pau-7A* explaining 12%–13% variation, and one QTL for seed Zn on chromosome 7A, QZn.pau-7A explaining 19% variation	Singh et al. 2010a
Two QTLs for Fe on chromosomes 2A and 7A, mapped at *Xwmc382–Xbarc124* and *Xgwm473–Xbarc29*, explained 12%–13% variation; a QTL for Zn on chromosome 7A, mapped at *Xcfd31–Xcfa2049*, explained 19% variation	Tiwari et al. 2009
A QTL on chromosome 3D for seed-Fe and four QTLs on chromosomes 3D, 4B, 6B, and 7A for Zn concentrations; QTL for seed-Fe concentration colocated with a QTL for shoot-Fe concentration and seed weight, with alleles for high Fe concentration coming from the same parent	Genc et al. 2009
11 QTLs on chromosomes 2A, 5A, 6B, 7A, and 7B for Fe and 6 QTL on chromosomes 2A, 2B, 3A, 4B, 5A, 6A, 6B, 7A, and 7B for Zn; clusters of QTLs on chromosome 2A, 5A, 6B, and 7A for seed protein and minerals	Peleg et al. 2009
Four QTLs for Zn concentration and seven QTLs for Zn content, with concentration QTLs colocated with those of content QTLs, possible to improve both traits simultaneously	Shi et al. 2008

phenotypic variation (Stangoulis et al. 2007). In barley, specific markers have been developed for a high zinc QTL on chromosome 2H (Sadeghzadeh et al. 2010), while other QTLs have been identified by Lonergan et al. (2009).

Iron reductases are members of the protein super-family of flavocytochromes and function in roots to convert Fe from a plant unavailable form (ferric, Fe^{3+}) to an available form (ferrous, Fe^{2+}) that can be readily absorbed (Grusak 1995). IRA is known to vary with plant growth conditions (e.g., soil pH and available iron concentration) (Grusak 2000). Common bean genotypes with high seed-Fe showed high IRA than those with low seed-Fe, suggesting a link between root uptake and seed loading of Fe in common bean (Grusak 1994, 2000, 2002). More recently, Blair et al. (2010b) reported a single major QTL for IRA under Fe-limited conditions (1 μM) on LG b02, and another major QTL under Fe-sufficient conditions (15 μM) on LG b11 that was associated with several QTL for seed Fe in common bean. Thus, the QTL for IRA under Fe-limited conditions may be useful in environments where beans are grown in alkaline soils, while the QTL for IRA under Fe-sufficient conditions may be useful for selecting for enhanced seed nutritional quality (Blair et al. 2010b).

Wild emmer wheat germplasm harbors a rich allelic diversity, including for seed minerals (Xie and Nevo 2008). A major locus, *Gpc-B1* (a 250-kb locus) mapped as a simple Mendelian locus (Distelfeld et al. 2006), associated with increased seed-protein (38%), -Fe (18%), and -Zn (12%) concentrations from wild emmer wheat germplasm (*Triticum dicoccoides*), encodes a NAC transcription factor (*NAM-B1*) that accelerates senescence and increases nutrient remobilization from leaves to developing seeds (Uauy et al. 2006; Distelfeld et al. 2007). *Triticum turgidum* is another useful wild emmer germplasm for improving seed mineral concentration in wheat. Peleg et al. (2009) mapped 82 QTL for 10 seed minerals (LOD score range of 3–17), with most of the positive alleles contributed by wild emmer accession, G18-16, and many QTL for the same trait mapped to homoeologous positions, reflecting synteny between the A and B genomes. *TtNAM-B1* affecting seed-protein, -Fe, and -Zn originating from wild emmer wheat has been cloned (Distelfeld and Fahima 2007). Furthermore, Singh et al. (2010a) identified two QTL (*QFe.pau-2A* and *QFe.pau-7A*) for Fe and a QTL (*QZn.pau-7A*) for Zn, which they transferred into interspecific progenies involving *Aegilops kotschyi* and *Aegilops peregrine*, both UUSS genome species. Such progenies showed 60%–136% enhanced seed-Fe and -Zn concentrations and 50%–120% increased Fe and Zn contents per seed as compared to the control cultivar that was introgressed with these

QTL (Tiwari et al. 2010). The profiling of introgression using simple sequence repeats (SSRs), genomic *in situ* hybridization (GISH), and fluorescent *in situ* hybridization (FISH) analysis further confirmed the introgression of chromosome 2S, 2U, 7S, and 7U into these progenies (Singh et al. 2010a). More recently, genetic mapping identified five putative QTL for seed-Fe density and two QTL for seed-Zn density in pearl millet (Kumar et al. 2010b).

A number of QTL for seed phosphorus (P) and/or phytate concentrations have been reported in common bean, rice, sorghum, soybean, and wheat (Table 3.5), some with either major effects or colocated with QTL affecting seed-Fe or -Zn concentration. For example, a major QTL for P (19% variation) on b01 colocated with QTL accounting 34% variation each for seed Fe and Zn in common bean (Cichy et al. 2009). Furthermore, QTL for seed P or phytate concentration or content related to seed weight QTL on LGs b06, b07, and b10 (Blair et al. 2009c) or genes coding for candidate enzymes involved in phytic acid synthesis pathway and markers associated with each gene (Fileppi et al. 2010) have been mapped in common bean. In rice, the candidate gene for low-phytate mutant alleles and markers (LPA1_CAPS for *lpa1-1* and LPA1_InDel for *lpa1-2*) showed complete cosegregation with mutant phenotypes (Zhao et al. 2008). Furthermore, the two QTL for seed-Zn concentration on chromosomes 4A and 4D colocated with QTL for P concentration, while four QTL for seed-Zn content on chromosome 2D, 3A, and 4A colocated with the QTL for P content, reflecting positive correlation between the seed-Zn and -P concentrations (see Section VI.B), which may provide opportunities for simultaneous improvement in seed-P and -Zn density in wheat (Shi et al. 2008).

Two QTL mapped onto LGs L and N control low phytate in soybean line, CX 1834 (Walker et al. 2006; Gao et al. 2008). Further, Saghai-Maroof et al. (2009) mapped and sequenced a putative multidrug resistance-associated protein (MRP) gene on LG N that contributes to low-phytate phenotype in CX 1834. This A to T mutation provided a single nucleotide polymorphism (SNP) marker for introgressing the low-phytate QTL from CX 1834 into desired breeding lines (Saghai-Maroof et al. 2009).

More recently, genes coding for candidate enzymes involved in the phytic acid pathway have been mapped and identified markers associated with each gene (*PvMIPSs*, *PvMIPSv*, *PvIMP*, *PvMIK*, *PvIPK2*, *PvITPKα*, *PvITPKβ*, *PvIPK1*), which may represent a useful resource to select genetic variants with low-phytate trait in common bean (Fileppi et al. 2010). Furthermore, González et al. (2010) discovered how phytate is produced in plants, by solving the structure of the protein $InsP_5$ 2-Kinase (IP_5 2-K), a distant member of the IPK family,

Table 3.5. Summary of marker/QTL analysis of seed phosphorus (P) and phytate (Phyt) concentration in barley, common bean, rice, sorghum, soybean, and wheat covering a period from 2005 to 2010.

QTL summary	References
Barley	
Flanking markers mapped 3 low phytic acid mutant alleles: *lpa1* at EBmac415 and Msu21; *lpa2-1* at Bmag120 and AWBMS0022; *lpa678* at EBmac701 and Bmag714B	Oliver et al. 2009
Common bean	
Genes coding for candidate enzymes involved in phytic acid pathway mapped and markers associated with each gene (*PvMIPSs*, *PvMIPSv*, *PvIMP*, *PvMIK*, *PvIPK2*, *PvITPKα*, *PvITPKβ*, *PvIPK1*) identified	Fileppi et al. 2010
Eight QTL for P on six LGs, in an Andean × Andean bush bean cross, 11%–40% variation; two QTL for Phyt on two LGs, 17%–18% variation; a QTL for P (19% variation) on B1 colocated with QTLs accounting 34% variation each for Fe and Zn on same LG	Cichy et al. 2009
Six QTL for P on LG B2 and B6 and three QTL for Phyt on LG B6, with QTL for P or Phyt were related to seed weight QTL on LGs B6, B7, and B10 in an inter-gene pool mapping population	Blair et al. 2009b
Rice	
Mutant alleles of the low phyt homozygous lethal (*XS-lpa2-1*) and nonlethal (*XS-lpa-2-2*) mapped: *XS-lpa2-1* gene to a region on chromosome 3 between marker RM14360 and RM1332, where also located the rice orthologue (*OsMRP5*) of the maize *lpa1*; a single base pair change in the sixth exon of *XS-lpa2-1* and a 5-bp deletion in the first exon of *XS-lpa2-2* resulted these mutations	Xu et al. 2009
TIGR locus LOC_Os02g57400 identified as the candidate gene for mutant alleles: *lpa1-1* is a single base pair substitution mutation while *lpa1-2* involves a 1,475 bp fragment deletion; a CAPS marker (LPA1_CAPS) for *lpa1-1* and an InDel marker (LPA1_InDel) for *lpa1-2* confirmed complete segregation with LPA phenotypes	Zhao et al. 2008
A single base pair change resulted *lpa* mutant, N15-186, mapped at RM15875 and RM15907 on chromosome 3, which also harbor rice orthologue of maize *lpa3*; *lpa* N15-186 is a mutant allele of the rice *myo*-inositol kinase (*Os MIK*) gene	Kim et al. 2008

(*continued*)

Table 3.5 (*Continued*)

QTL summary	References
A common QTL for P and Phyt on chromosome 5, explained 24% variation	Stangoulis et al. 2007
Lpa1 locus (135 kb) fine mapped at RM3542 and RM482 and further delimited to a 47 kb region containing eight putative open reading frames	Andaya and Tai 2005
Soybean	
A locus on LG N near *Satt237* accounted for 41% variation in Pi, which is inversely related with Phyt, while another locus near *Satt237* on LG L explained 11% variation and interaction between the two loci accounted additional 8%–11% variation	Walker et al. 2006
Wheat	
Four QTL for P concentration and six QTL for P content; two QTL for Zn concentration on chromosomes 4A and 4D colocated with the QTLs for P concentration, while four QTL for Zn content on 2D, 3A, and 4A colocated with QTLs for P contents	Shi et al. 2008

which represents a key point in the metabolism of highly phosphorylated inositols, and could be a valuable tool in plant physiology to design low-phytate crops.

D. QTL Mapping, Cloning, and Introgression of β-Carotene into Adapted Germplasm

The natural plant pigments, termed as carotenoids, which are fat soluble, are important source of vitamin A and antioxidants. The two groups of carotenoids are provitamin A and nonprovitamin A carotenoids. The former includes three carotenoids, namely β-carotene, β-cryptoxanthin, and α-carotene, all precursor for vitamin A, while the latter consists of lutein and zeaxanthin, which act as antioxidants. Of all the carotenoids, β-carotene is the most widely distributed in plants and the one most efficiently converted to vitamin A. The consumption of carotenoid-rich foods is associated with reduced risks of developing cancer and cardiovascular diseases, enhanced immune responses, improved vision, and prevention of night blindness as well the maintenance of healthy skin and gastrointestinal or respiratory systems. Carotenoids in plants play a crucial role in photosynthesis, membrane stability, growth, and development (Menkir et al. 2008).

Unlike other agronomically important traits, only limited germplasm sets have been assessed for β-carotene, predominantly in chickpea, maize, pearl millet, sorghum, and wheat (Table 3.6), with some lines accumulating β-carotene as high as 7.6 $\mu g\, g^{-1}$ in pearl millet (Hash et al. 1997) and 15.6 $\mu g\, g^{-1}$ in maize (Harjes et al. 2008; Menkir et al. 2008). Genotypic differences for β-carotene have also been reported in rice: some germplasm had shown β-carotene in unpolished seeds while others having no β-carotene in unpolished seeds. The rice germplasm with β-carotene in unpolished seeds include Amarillo Cuba, Dudemasino, Sirendah Kuning, Bongkitan, Calibo, Khao Dawk Mali 105, and Klemas (Tan et al. 2005). Furthermore, Kandlakunta et al. (2008) in a comprehensive study involving major cereals and legumes and commonly consumed vegetables detected high β-carotene in chickpea (15.7 $\mu g\, g^{-1}$), green gram (12.8 $\mu g\, g^{-1}$), red gram (12.4 $\mu g\, g^{-1}$), and maize (17.1 $\mu g\, g^{-1}$), while among vegetables, they detected high β-carotene in yellow pumpkin (118 $\mu g\, g^{-1}$), green chillies (102 $\mu g\, g^{-1}$), field beans (55.4 $\mu g\, g^{-1}$), French bean (39.3 $\mu g\, g^{-1}$), ridge gourd (32.4 $\mu g\, g^{-1}$), green beans (23.9 $\mu g\, g^{-1}$), and brinjal (16.9 $\mu g\, g^{-1}$).

Maize is an important source of provitamin A (β-carotene, α-carotene, and β-cryptoxanthin) and the nonprovitamin A including lutein and zeaxanthin. Most of the yellow maize grains consumed worldwide have

Table 3.6. Natural genetic variation for β-carotene in chickpea, maize, pearl millet, sorghum, and wheat covering a period from 1962 to 2009.

No. of germplasm evaluated	Summary of the variation in β-carotene concentration ($\mu g\, g^{-1}$ seed)	Range variation in β-carotene concentration ($\mu g\, g^{-1}$ seed)	References
Chickpea			
Two lines	0.09–0.48	0.37	Abbo et al. 2005
Maize			
155 Inbreds	0.13–1.98	1.85	Yang et al. 2010
228 Inbred	0.06–13.6	13.54	Harjes et al. 2008
233 RILs and parental lines	0.05–2.04	1.99	Chander et al. 2008
421 Yellow endosperm inbreds	0.3–15.6	15.3	Menkir et al. 2008
17 Tropical varieties	0.45–2.18	1.73	Menkir and Maziya-Dixon 2004
10 Inbreds	0.56–2.40	1.84	Egesel et al. 2003
125 Inbreds	From traces to 7.3	7.3	Quackenbush et al. 1963
109 Yellow endosperm lines	0.5–5.1	4.6	Brunson and Quackenbush 1962
Pearl millet			
IP 15533 and IP15336	6.1–13.7	7.6	Hash et al. 1997
13 Lines	0.23–0.63	0.4	Khangura et al. 1980
Sorghum			
10 Carotenoids diversity panel accessions	0.10–0.22	0.12	Salas Fernandez et al. 2009
KS115 and Macia	0.025–0.074	0.049	Salas Fernandez et al. 2008
RILs (KS115 × Macia)	0.003–0.148	0.145	
82 Including yellow endosperm lines	0.56–1.13	0.57	Reddy et al. 2005
10 Lines	0.22–3.23	3.01	Worzella et al. 1965
Wheat			
13 Lines	0.03–o.13	0.10	Ramachandran et al. 2010
5 Lines	3.0–8.5a	5.5[z]	Santra et al. 2005

[z]Part per million.

only 0.5–1.5 $\mu g\, g^{-1}$ β-carotene. Maize has been extensively studied for molecular polymorphism, mapped QTL, chemical pathways, and genes involved in carotenoids biosynthesis, including β-carotene (Table 3.7). Carotenes are intermediates in the carotenoids biosynthetic pathway and lycopene (the immediate precursor of provitamin A carotenes) represents a branch point in this pathway, which is further modified by lycopene β-cyclase and lycopene ε-cyclase (LCYE) enzymes that catalyze formation of terminal β- and ε-rings, respectively, to form either β-carotene, α-carotene, or β-cryptoxanthin. This pathway continues with hydroxylation of the carotenes that depletes the provitamin A pool by converting these compounds to nonprovitamin A xanthophylls. Pathway branching and hydroxylation are therefore key determinants in controlling vitamin A levels. Polymorphism at the *LCYE* locus in maize explained 58% of the variation in α- and β-carotene and a threefold difference in provitamin A compounds (Harjes et al. 2008), while a rare genetic variant, *β-carotene hydroxylase 1* (crtRB1), increases β-carotene substantially in maize grains (Yan et al. 2010). Further, metabolite sorting of a germplasm collection identified 10 genetically diverse subsets representing biochemical extremes for maize kernel carotenoids and transcript profiling of this subset led to the discovery of the *Hydroxylase 3* locus that coincidently mapped to a carotene QTL (Chander et al. 2008). The natural alleles at *Hydroxylase 3* locus contribute 78% of variation and approximately 11-fold differences in β-carotene relative to β-cryptoxanthin and 36% of the variation and fourfold difference in absolute levels of β-carotene (Harjes et al. 2008). The reduction in *HYD3* transcripts leads to reduced conversion of β-carotene to downstream xanthophylls, causing β-carotene to accumulate (Vallabhaneni et al. 2009). Genetics tests such as the *HYD3* assay (Vallabhaneni et al. 2009) together with the previously described *LCYE* assay (Harjes et al. 2008) may be used to select germplasm containing optimal *HYD3* and *LCYE* alleles in breeding programs, which will lead to higher β-carotene levels in maize endosperm when both genes are highly expressed than either with optimal alleles of either gene alone. Furthermore, the experimental evidence from the association and linkage mapping reveals that *crtRB1* underlies a principal QTL associated with β-carotene concentration and conversion in maize kernels and *crtRB1* alleles associated with reduced transcript expressions correlate well with higher β-carotene (Yan et al. 2010). The most favorable *crtRB1* alleles that are rare in frequency and unique to temperate germplasm are being introgressed via inexpensive PCR-marker-assisted selection into tropical maize germplasm adapted to developing countries. A program at CIMMYT has already achieved

Table 3.7. QTL associated with β-carotene in chickpea, maize, and sorghum covering a period from 2004 to 2010.

QTL summary	Reference
Chickpea	
Four QTL associated with β-carotene	Abbo et al. 2005
Maize	
A gene encoding β-carotene hydroxylase 1 (*crtRB1*) underlies a major QTL associated with β-carotene concentration and conversion; *crtRB1* alleles associated with reduced transcript expression correlated with higher β-carotene concentration	Yan et al. 2010
Cloned/characterized four cDNAs encoding carotenogenic enzymes, two encoding CRTISO (*ZmCRISO1* and *ZmCRISO2*) and two encoding BCH (*ZmBCH1* and *ZmBCH2*), mapped on different chromosomes; all four genes expressed during endosperm development and mRNA levels increased with carotenoids accumulation until 25DAP except for *Zmcrtiso2* mRNA levels which remains high for another 5 days while the carotenoids content continues to increase	Li et al. 2010a
Hydroxylase3 locus mapped to a carotene QTL, with three alleles contributed 78% variation and 11-fold differences in β-carotene relative to β-cryptoxanthin and 36% variation and fourfold difference in absolute β-carotene	Vallabhaneni et al. 2009
31 QTL for total carotenoids on seven chromosomes, much of the variation explained by two loci *y1* and *y9*; candidate gene *phytoen synthase 1* (*psy1*) marker "Y1ssr" tightly linked to a major QTL explaining 7%–27% variation for carotenoids	Chander et al. 2008
Variation at *lycopene epsilon cyclase* (*lcyE*) locus alters flux down α-carotene versus β-carotene branches of the carotenoids pathway, with four natural *lcyE* polymorphism explained 58% of the variation in α-carotene and β-carotene and threefold difference in provitamin A compounds	Harjes et al. 2008
A major QTL for both β-carotene and β-cryptoxanthin on chromosome 6 and 7; genes *y1*, associated with phytoene synthase, and *vp9*, associated with ζ-carotene desaturase, linked with observed variation in carotenoids in maize and also with carotenoids variation in Solanaceae	Wong et al. 2004
Sorghum	
Color QTL significantly correlated with levels of all carotenoids, and color QTL colocalized with carotenoids QTL; β-carotene QTL (*Bc-1.1*, *Bc-2.1*, *Bc-2.2*, *Bc-2.3*, *Bc-10b.1*) mapped on chromosomes 1, 2, and 10 explained 8%–15% variation, with *Bc-2.2* (11.6% variation) remained stable across environments, and located close to *Psy3* gene involved in carotenoid biosynthesis pathway	Salas Fernandez et al. 2008

HarvestPlus provitamin A target concentrations by introgressing favorable *crtRB1* and *lcyε* alleles into tropical maize germplasm in a number of breeding lines, including a high β-carotene (15 $\mu g\,g^{-1}$) temperate hybrid, CI7 × DEexp that contains the most favorable *crtRB1* alleles (Yan et al. 2010; Pixley et al. 2011b). Several hybrids with β-carotene concentration between 5 and 8 $\mu g\,g^{-1}$ and agronomically competitive with commercial hybrids were tested during summer 2009/2010 in Zambia and Zimbabwe, and the best hybrids will be further evaluated in Mexico, Zambia, and Zimbabwe prior to their release in these countries (Pixley et al. 2011b). Likewise, the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, has introduced high β-carotene trait from temperate maize to tropical maize inbred lines, which ranged from 2.5 μg^{-1} to 10.5 μg^{-1}, and hybrids involving some of these inbreds showed 25%–79% more provitamin A concentration than Oba Super II, a commercial yellow hybrids widely grown in Nigeria. The grain yield and agronomic traits of the best hybrids were comparable to those of Oba Super II (IITA annual report, 2009/2010; http://annualreport.iita.org/?p=481). The University of Illinois, USA has also reported some of the high provitamin A lines of maize that include A 619, C 17, DE 3, and SC 55 (see supplementary Table 1, Yan et al. 2010). The researchers at Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora, India and other affiliated Indian Council of Agricultural Research (ICAR) institutions in India have identified few promising maize inbred lines (CM 136 and CM 138, 08 HPLET-03-8 and 08 HPLET-03-41, NAI 125, BAJIM-8-10 and SE 547), with total carotenoids ranging from 20.2 to 56.1 $\mu g\,g^{-1}$ seed and β-carotene from 10.6 to 14.9 $\mu g\,g^{-1}$ seed (P.K. Agrawal, VKPAS, pers. commun.).

The 'Golden Rice 2' genetic stocks have been used to introgress high β-carotene trait into several Asian rice cultivars, both *japonica* and *indica* types, and it is expected that the products from such introgressions will soon be available for national release in Bangladesh, India, Indonesia, and the Philippines (Barry 2011).

Sorghum landraces have shown significant variation for carotenoids, with lutein, zeaxanthin, and β-carotene the predominant carotenoids. Yellow-endosperm color QTL in sorghum colocalized with carotenoid QTL, with major β-carotene QTL *Bc-2.2* found close to *Psy3* gene, which is significantly associated with β-carotene concentration and endosperm color (Salas Fernandez et al. 2008). Furthermore, Salas Fernandez et al. (2009) reported that 164 yellow endosperm landraces from Niger and Nigeria clustered separately from the genotypes in a 68 individual diversity panel (Casa et al. 2008) with accessions differing in geographic origin and carotenoids content, which may provide

additional source of genetic diversity to breed for β-carotene content in this crop.

The first step toward understanding how carotenoids are synthesized is to identify the enzymes involved and isolate the corresponding genes. Several maize complementary deoxyribonucleic acids (cDNAs) encoding carotenogenic enzymes have been cloned and genes identified: *psy1*, *psy2*, and *psy3* (phytoene synthase), *pds* (phytoene desaturase), *zds* (ζ-carotene desaturase), *lcyb* (lycopene β-cyclase), and *LCYE* (Li et al. 2010a). Further, Li et al. (2010a) cloned and characterized four additional cDNAs, two each representing carotenoids isomerase (CRTISO) and β-carotene hydroxylase (BCH): *Zmcrtiso1* and *Zmcrtiso2* for CRTISO and *Zmbch1* and *Zmbch2* for BCH, mapped to different chromosomes. All four genes expressed during endosperm development and the coordinated up-regulation of *Zmcrtiso1*, *Zmcrtiso2*, *Zmbch1*, *Zmbch2* until 25 days after pollination is consistent with the observed accumulation of carotenoids, although *Zmcrtiso2* remained at high levels for the next 5 days while the carotenoids content continues to increase. The enzymes are highly conserved in sequence, expression, and activity, but subtle differences in the expression profiles of the CRTISO enzymes and the expression and activities of the BCH enzymes hint at divergent roles in plant carotenoids biosynthesis that may be useful in the development of more refined strategies to engineer carotenoids synthesis and composition in staple crops (Li et al. 2010a) (Fig. 3.1).

E. Developing Seed Iron- and Zinc-Dense Cultivars Using Conventional Breeding and Genomic Tools and Cultivars Adoption

1. Grain Minerals-Dense Cultivars and Hybrids. Unlike other approaches (such as fortification or supplementation), biofortification is a sustainable and cost-effective way to address malnutrition in developing countries. The main advantage with this approach is that once seed mineral-dense cultivars are developed, adapted for cultivation, and accepted by the end users, these will be grown by the farmers and the produce from such mineral-dense crops will be easily available to large communities at low cost–even in remote regions where other approaches may not succeed. Until recently, breeding efforts focused on raising productivity with hardly any thought to improving seed quality except for improving the protein quality in maize and wheat, and more particularly the essential seed-micronutrients such as Fe and Zn. Technological innovations including the use of dwarfing genes have

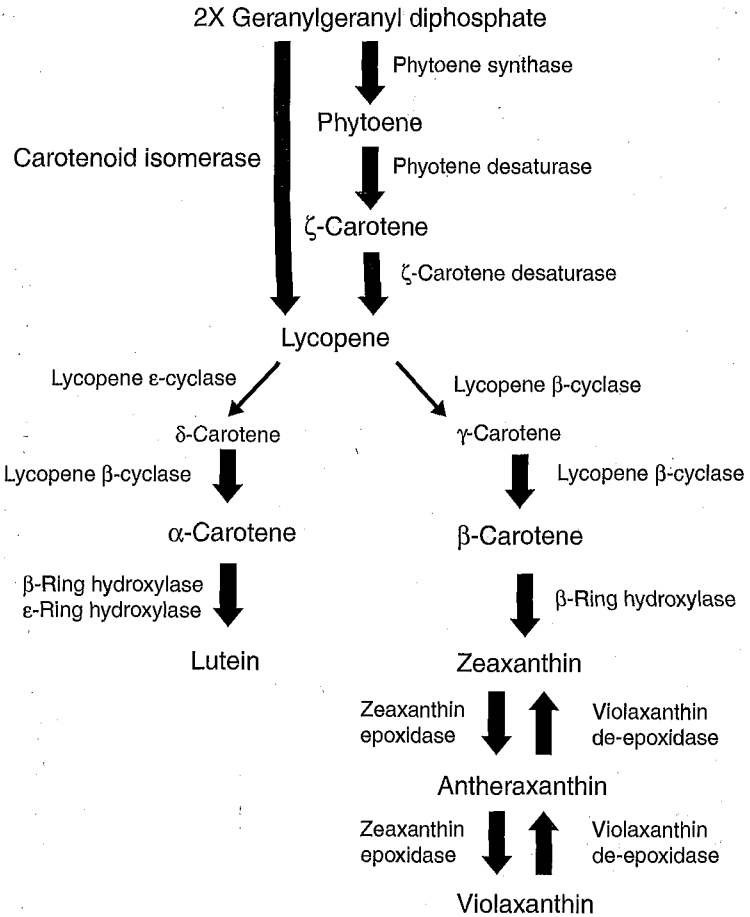

Fig. 3.1. Plant carotenoid biosynthesis pathway. (Adapted from Ramachandran et al. 2010).

boosted crop yields across continents; with the result that many countries are now more or less self-sufficient in food production. What is needed now is to improve the nutritional quality of staple food crops to provide nutritious foods to the poor who often consume diets poor in micronutrients. Agriculture research must therefore focus on both increasing production and improving diets in sustainable ways. Breakthroughs in plant breeding and nutritional genomics including transgenes (see Section VII.A) has simplified and hasten the development of seed mineral-dense cultivars to address rampant micronutrient malnutrition in developing countries.

Breeding for seed mineral-dense cultivars is a recent activity (since mid-1995), mostly supported by the HarvestPlus Challenge Program, to develop β-carotene (precursor of vitamin A) rich and/or mineral-dense (largely Fe and Zn) cultivars of six major staple food crops (Phase I)–common bean, cassava, maize, rice, sweet potato, and wheat. Ten additional crops (bananas/plantains, barley, cowpeas, peanut, lentil, millet, pigeon pea, potatoes, sorghum, and yam) were added in Phase II to improve seed mineral density (Pfeiffer and McClafferty 2007).

The proof of concept was demonstrated by CGIAR institutions, showing that high nutrient traits can be combined with superior agronomic characteristics into high yielding genetic backgrounds using conventional breeding, applied genomics, and/or transgene(s) technology. CGIAR institutions in partnership with National Agricultural Research System (NARS) have made considerable progress toward developing seed mineral (iron and/or zinc)-dense elite germplasm/cultivars in common bean, rice, and wheat (Table 3.8). For example, breeding at CIAT is directed to transfer high seed Fe and Zn traits into a range of commercial classes in both gene pools of common bean, that is, large-seeded Andean and small-seeded Mesoamerican beans. A number of donor lines with high seed Fe and Zn were crossed with adapted cultivars from different market and seed types and the progenies with high seed-Fe and -Zn concentrations are in various stages of evaluation. The variation observed in some of the progenies ranged from 76 to 154 ppm Fe and from 28 to 44 ppm Zn, with some progenies combining high Fe and Zn into improved genetic background. Micronutrient-dense bush and climbing bean cultivars with high yield potential have already been released in Bolivia, Colombia, and Zimbabwe (Blair et al. 2010d), and others are in prerelease stage in several Eastern African countries (e.g., Rwanda, DR Congo).

The first seed Fe-dense rice breeding line, IR68144-2B-2-2-3, has been released as Maligaya Special 13 (MS13) for cultivation in the Philippines (Sison et al. 2006). An improved version of the open pollinated pearl millet variety, ICTP 8203, containing ~10% higher seed-Fe than the original ICTP 8203 background, has been identified for multilocational evaluation prior to its release in India, with adaptation to most of peninsular India. ICTP 8203 was produced by random mating five S_2 progenies of an *Iniari* (early maturing) landrace originating from Northern Togo, and released as MP124 for cultivation in Maharashtra and Andhra Pradesh, India. It flowers in 50–52 days; the panicles are of medium length, compact to semicompact, and cylindrical to lanceolate with a slight tapering toward the tip; dark gray and large seed size (>12 g 1000^{-1}); and resistance to downy mildew caused by *Sclerospora*

Table 3.8. Seed-iron (Fe) and/or -zinc (Zn) dense advanced breeding lines/cultivars developed in common bean, pearl millet, rice, and wheat covering a period from 2003 to 2010.

Summary of the seed-Fe and/or -Zn dense lines developed by conventional crossing and selection	References
Seed-Fe concentration	
Common bean	
NUA35 and NUA56 showed on average 18 and 23 mg kg^{-1} higher Fe than control cultivar 'CAL96' (58 mg kg^{-1}), when evaluated over 15 sites with diverse agroecologies in Bolivia, Colombia, Costa Rica, and Guatemala	Blair et al. 2010d
Pearl millet	
Improved versions of open pollinated variety (ICTP 8203) outyielded original ICTP 8203 bulk (2874 kg ha^{-1}) by 7%–10%, with 7%–18% more Fe (94–103 mg kg^{-1}) and 5%–15% more Zn (63–69 mg kg^{-1})	ICRISAT 2010
Rice	
An seed-Fe dense line, IR68144-2B-2-2-3, released as Maligaya Special 13 (MS13) in Philippines	Sison et al. 2006
Wheat	
A synthetic line, 68.111/RGB-4//WARD/3/FGO/4/RABI/5/Ae. Sq. (878), showed 25% more Fe than controls	Calderini and Ortiz-Monasterio 2003
Backcross-derived lines involving *A. kotschyi* showed 67%–116% increase in Fe than control, WL711 (22 mg kg^{-1} seed)	Tiwari et al. 2010
Amphidiploids involving *A. kotschyi* accessions showed 97%–117% and 68%–111%, respectively, higher Fe in seed and flag leaf than controls	Rawat et al. 2009a
Seed-Zn concentration	
Common bean	
Progenies involving Perola × Guapo Brilhante or TPS Nobre × Guapo Brilhante recorded 37% increase in Zn content	de Rosa et al. 2010
NUA35 and NUA56 showed on average 8 and 7 mg kg^{-1} higher zinc than control cultivar 'CAL96' (26 mg kg^{-1}), when evaluated over 15 sites with diverse agroecologies in Bolivia, Colombia, Costa Rica, and Guatemala	Blair et al. 2010d
Progenies with 10% increase in Zn content developed	Gelin et al. 2007
Wheat	
A synthetic line, 68.111/RGB-4//WARD/3/FGO/4/RABI/5/Ae. Sq. (878), showed 25% more Zn than controls	Calderini and Ortiz-Monasterio 2003
Backcross-derived lines involving *A. kotschyi* showed 75%–133% increase in Zn than control, WL711 (20 mg kg^{-1} seed)	Tiwari et al. 2010
Amphidiploids involving *A. kotschyi* accessions showed, respectively, 67%–139% and 54%–91% higher Zn in seed and flag leaf than controls	Rawat et al. 2009a

graminicola (Rai et al. 1990). Further work at ICRISAT is in progress to identify genetically diverse germplasm (noniniary type) from the recently constituted pearl millet mini-core collection (Upadhyaya et al. 2010) for developing hybrid parental lines with high seed-Fe concentration. In a two-rowed barley, the pooling of three most favourable QTL alleles increased seed-Zn content and concentration by an average of 53% and 75%, respectively (Lonergan et al. 2009).

The AgroSalud project (www.AgroSalud.org), a consortium of centers from the CGIAR, NARS, Universities, and NGOs formed in 2005 with funding support from Canadian International Development Agency (CIDA), aims to improve food and nutrition security among vulnerable populations living in 14 Latin American and Caribbean countries through nutritionally enhanced biofortified crops (beans, maize, rice, sweet potato) and food products derived from these crops and cassava. To date, *AgroSalud* have commercially released 21 maize cultivars with higher tryptophan and lysine levels (protein quality not the subject of this review) in Bolivia, Colombia, El Salvador, Guatemala, Haiti, Honduras, Mexico, Nicaragua, and Panama; eight rice cultivars with higher iron in Bolivia, Cuba, and Panama; five bean cultivars with higher iron in Bolivia, Brazil, Cuba, and Guatemala; and eight sweet potato cultivars with more β-carotene in Brazil, Cuba, Dominican Republic, Haiti, and Peru. An additional 10 nutritionally enhanced cultivars are in the pipeline for release in the region. More importantly, the project partners in Cuba, Nicaragua, and Panama have succeeded in influencing the policy makers to include biofortification as a core breeding activity for improving the nutritional quality of these crops (http://www.AgroSalud.org/descargas/AgroSalud_consolidated%20final_report_2010_ene18_11.pdf).

More recently, Glahn and Hoekenga (2011) detected three QTL with large effects for bioavailable iron in maize that they integrated into new cultivars of maize highly similar to each other but containing different QTL for iron bioavailability. Further, the feeding trials demonstrated that QTL associated with high bioavailable iron in maize provided approximately twice as much iron to the birds relative to the low bioavailable iron lines.

Wild and weedy or uncommonly cultivated relatives of common bean and wheat have shown large variability for seed-Fe and -Zn concentrations (see Section V.A). Mineral nutrient concentrations in the perennial lines derived from an interspecific cross involving a tall wheatgrass (*Thinopyrum elongatum*) and bread wheat revealed 44%, 40%, 24%, 23%, 32%, 30%, and 30% higher than control cultivars for calcium, copper, iron, magnesium, manganese, phosphorus, and zinc, respectively (Murphy et al. 2009). Interspecific crosses involving wild emmer and *Aegilops* species

with cultivated wheat's produced seed mineral (Fe and Zn)-dense progenies (Rawat et al. 2009b; Neelam et al. 2010a; Tiwari et al. 2010). For example, backcross progenies involving *Aegilops peregrina* ($2n = 28$, $U^PU^PS^PS^P$) with *T. aestivum* showed significantly higher seed micronutrients (nearly 100% increase in seed Fe and more than 200% increase in seed Zn), high thousand-seed weight, and harvest index. The seed-Fe concentration in wheat cultivars was ~28 mg kg^{-1} while zinc ~22 mg kg^{-1} seed. The *in situ* hybridization and marker analysis of these progenies further revealed that chromosomes 4 and 7 of *Ae. peregrina* carry the genes for high seed-Fe and -Zn concentrations. Likewise, CIAT conducted interspecific backcrosses and recurrent selection to transfer high seed-Fe and -Zn from *Phaseolus coccineus, P. dumosus,* and *P. acutifolius* into common bean. Some interspecific progenies showed 30%–40% increases in seed Fe over the control cultivars (Acosta-Gallegos et al. 2007; Steve Beebe, CIAT, pers. commun. 2011).

Marker-assisted backcrossing (MABC) has been successfully employed to transfer the low-phytate trait into improved genetic backgrounds in soybean. Landau-Ellis and Pantalone (2009) used SSRs (Satt237 on LG N and Satt561 on LG L) (Walker et al. 2006) to transfer the low-phytate trait from CX 1834-1-2, which is controlled by two recessive genes (Oltmans et al. 2004), into an improved genetic background, and by BC_4 generation, they fully recovered the recurrent parent genome of 5601T (soybean cultivar of maturity group V) while simultaneously confirming the presence of both low-phytate loci in backcross progenies. Evidence suggests, therefore, that the MABC approach is an effective breeding method to transfer low-phytate trait into improved genetic background.

2. Adoption of Biofortified Cultivars by Farmers. Adoption studies conducted under AgroSalud project suggest high acceptance of nutritionally enhanced cultivars of maize (tryptophane) in El Salvador, beans (Fe) in Cuba and Nicaragua, sweet potato (β-carotene) in Nicaragua, and rice (Fe) in Bolivia and Panama (www.AgroSalud.org/descargas/poster%20agrosalud_ingles_v2_impresion_oct10.pdf). The pattern of early diffusion and adoption of β-carotene-rich cassava cultivars (Fukuda et al. 2008) in northeast Brazil revealed large gap between actual early adoption in some regions (62.5% vs. 15%) in comparison with the potential adoption (62% and 64%), which González et al. (2011) relate it with public awareness of the new cultivars advantages, public entities as the main information sources, and farmers participation in early stages of evaluation. This study also revealed the lack of seed availability as one of the main factors limiting the adoption process and thus, suggested strengthening seed production and distribution for better

diffusion of the newly developed cultivars among the producers (González et al. 2011). Preliminary studies in Kenya, Mozambique, and South Africa have shown that provitamin A biofortified maize (grain color orange yellow because of β-carotene) may be adopted where white-seeded maize is the predominant staple crop (Wolson 2007; Stevens and Winter-Nelson 2008).

QPM is the first biofortified crop, released and disseminated in East Africa. The adoption of QPM varied greatly in Ethiopia, Kenya, Tanzania, and Uganda, from none in Kenya to more than half of the farmers growing QPM in Uganda. Further, analysis of the pattern of adoption of QPM in these countries revealed that agronomic performance of QPM varieties; postharvest processing; the cooking and sensory characteristics (taste and flavor); understanding QPM's nutritional benefits; higher participation of the farmers in QPM evaluation; and reliable and continuous supply of seeds, all significantly impacted the adoption of QPM in Tanzania and Uganda (Hugo de Groote, pers. commun., CIMMYT). A QPM cultivar, Obatanpa, first released in Ghana in 1992, is now commercially grown in 15 other African countries (Krivanek et al. 2007).

A range of biofortified crops (Fe, Zn, and/or β-carotene) are being developed, many in pipeline, which will soon be available to farmers for cultivation that will provide opportunities to the social/economic personal to assess adoption and to nutritionists to assess the impact of these cultivars on human nutrition leading to improved health in regions prone to micronutrient malnutrition.

F. Breeding Issues Associated with Selecting Seed Mineral-Dense Progenies

Mineral concentration in a plant's shoot is dependent on the rate of dry matter accumulation and micronutrients absorption. When dry-matter accumulation increases at a faster rate than micronutrient accumulation, a so-called "dilution effect" can be observed, whereas increased micronutrient concentrations will result when micronutrient accumulation increases at a faster rate than dry matter accumulation ("synergism effect") (Jarrell and Beverly 1981). It is a well-known fact that when breeders select for one-resource using trait, such as yield, less resources remain for other resource-using functions, that is, trade-offs between seed size and seed number, yield and resistance to pest and diseases, or yield and nutrition (Davis 2005, 2009; Morris and Sands 2006). The extent of such trade-offs is a question that must be addressed.

Modern plant breeding has revolutionized agriculture, resulting in several fold increases in production and productivity of most staple

crops that form the major basis for human diets. To achieve higher productivity, plant breeders have concentrated mainly on traits that raise yield per se. The so-called "green revolution" has raised productivity, but did not concentrate on producing food that was as nutritious and tasty as from the traditional cultivars/landraces. For example, declines are reported in the mineral density of broccoli (Farnham et al. 2000) and wheat (Garvin et al. 2006; Fan et al. 2008; Murphy et al. 2008), and in protein concentration of maize (Scott et al. 2006). The evidence for these crops clearly shows uniformly inverse associations between seed yield and nutrition, indicating that genetic dilution effects (trade-offs) may be common when selective breeding successfully increases crop yields (Davis 2009).

Furthermore, nutritious germplasm/cultivars can be more vulnerable to pests and diseases (Arnason et al. 1993, Morris and Sands 2006). The environmental trade-offs between yield and nutritional quality could result either from the variation in soil health or quality (nutrient-deficient soils or soils affected by salinity/alkalinity or acidity; such soil conditions lead to nutrient imbalance in the soil) and soil fertility related factors (i.e., NPK effect on seed composition) or due to drought and high temperature during the seed development. For example, reduced seed protein and minerals and altered lipid composition have been reported in barley, potato, rice, and soybean as a consequence of nitrogenous fertilizer application (Riedel 2010) or global climatic changes, especially due to high temperature and high CO_2 concentrations in the environments (Högy and Fangmeier 2008; Taub and Wang 2008; Taub et al. 2008; Pleijel and Danielsson 2009; Sinha et al. 2009; DaMatta et al. 2010; Erbs et al. 2010). The challenge for the agricultural research community is to minimize any possible negative trade-offs between yield and nutrient concentrations, to provide nutritious staple foods for growing populations (Davis 2009).

How much seed phytate can be reduced (either through mutagenesis, or conventional crossing, and selection or by using transgenes) without adverse effect on seed germination and plant development is an open question. Research to date suggest that it is possible to breed first-generation low-phytate (50%–95% reduction) hybrids/cultivars in maize, barley, rice, and soybean, which performed relatively better than the original low-phytate mutants derived from the use of mutagens (Raboy et al. 2001; Raboy 2002; Spear and Fehr 2007); however, it is yet to be investigated whether low-phytate seeds remain beneficial to human health?

In conclusion, few QTLs with major effects on seed Fe, Zn, and phytate have been reported in common bean. A major QTL for IRA

under Fe-deficient and another major QTL for IRA under Fe-sufficient conditions have been identified, the latter associated with several QTL for seed-Fe concentration. A major locus from wild emmer wheat, *Gpbc-B1*, mapped as a single Mendelian locus encodes a NAC transcription factor, *TtNAM-B1*, has been cloned, which accelerates senescence and increases seed protein, Fe, and Zn. Interspecific progenies containing two Fe QTL and one Zn QTL from *Aegilops* species showed exceptionally high seed-Fe and -Zn concentrations. Yellow endosperm color QTL in sorghum is colocalized with carotenoids QTL, with major β-carotene QTL *Bc-2.2* found close to *Psy3* gene, which is significantly associated with β-carotene concentration and endosperm color.

AgroSalud project has released several maize cultivars with high tryptophan and lysine; beans and rice with high seed Fe; and β-carotene-rich sweet potato have been released for cultivation in several Latin American countries. CIAT has released seed micronutrient-dense (Fe and Zn) common bean in Bolivia, Colombia, and Zimbabwe; with many in prerelease stage in several Eastern African countries including those in Rwanda and DR Congo. In Asia, a seed iron-dense rice cultivar has been released for cultivation in the Philippines, while an improved version of an open pollinated variety with high seed iron, ICTP 8203, is in the prerelease stage in India.

CIMMYT and IITA researchers have identified β-carotene-rich maize germplasm and transferred high β-carotene trait into improved genetic background. The selected lines are under multilocational evaluation prior to their release in Mexico and in Africa. At the CIMMYT, molecular markers for *LycE* and *HydB* have accelerated breeding by one season, and have substantially enhanced efficiency and effectiveness of high-provitamin A maize breeding. Marker-assisted selection has been successfully employed to transfer low phytate into improved soybean cultivars.

Limited studies on cultivar adoption suggest high adoption of nutritionally enhanced crops in some countries in Latin America, which brought a paradigm shift to include biofortification as core breeding activity to enhance the nutritional quality of these crops in Cuba, Nicaragua, and Panama.

VII. ENHANCING SEED IRON, ZINC, AND B-CAROTENE USING TRANSGENE(S)

A. Transgenes for Nutritional Enhancement of Food Crops

Targeted improvement of mineral nutrition through plant biotechnology may be another more sustainable albeit sometimes controversial

approach to combat widespread deficiencies in human populations, particularly in developing world (Zimmerman and Hurrel 2002; Bouis 2007; Hirschi 2009). Few genes have been identified that either associate with increased Fe and Zn uptake/accumulation in developing seeds or reduced phytate such as the enzyme phytase that degrades phytate and thus releases more bioavailable Fe and Zn to absorption. The biosynthetic pathway to carotenoids synthesis and genes associated with accumulation of carotenoids has been characterized in several related species (Misawa et al. 1990; Zhu et al. 2007; Lu and Li 2008). We summarize below the update on use of transgene(s) to enhancing seed Fe, Zn, and/or β-carotene or reducing phytate concentrations in maize and rice, two of the three major cereal seeds, and soybean, an oil crop but rich source of protein.

1. Iron and Zinc. Ferritin is the iron storage protein found in animals, plants, and bacteria, which can carry up to 4,500 iron atoms in a central cavity (Theil 1987). The ferritin protein takes up Fe, stores it in a nontoxic form, and releases it when needed for metabolic functions. The ferritin iron represents a form of Fe that is highly bioavailable to humans. Plant ferritins are the products of a small gene family. Plant ferritin genes have been obtained from common bean, cowpea, lentil, maize, pea, and soybean (Prasad and Nirupa 2007). The expression of *ferritin* gene either from soybean or common bean, driven by the endosperm-specific promoters, have led to two- to threefold higher seed-Fe and -Zn levels in transgenic brown and/or polished seeds than WT rice (Table 3.9). Ferritins are the major players in plant Fe homeostasis. However, the use of ferritin as a biotechnology target to enrich seeds with Fe has met with limited success (only 1.5- to 3-fold increase compared to WT), probably due to a posttranscriptional regulation of the seed ferritin protein during fruit maturation.

A recent study in *Arabidopsis* reveals that there is no specificity for metal loading into the fruit, and that the step controlling metal loading into the seed occurs most likely by the regulation of hull to seed metal transport. Thus, the success of ferritin overexpression strategies for Fe biofortification would strongly benefit from the identification and engineering of mechanisms enabling the translocation of high amounts of Fe into the seed (Ravet et al. 2009).

In this regard, another gene for *Nicotianamine synthase* (NAS), a chelator of metals, is ubiquitous in higher plants and is a key component of their metal assimilation and homeostasis. Manipulation of cellular nicotianamine (NA) concentrations is a possible approach to improving Fe concentrations *in planta* (Douchkov et al. 2005). Overexpressing

Table 3.9. Examples of transgenic rice with enhanced seed-iron (Fe) and -zinc (Zn) concentrations covering a period from 1999 to 2010.

Gene	Source	Promoter	Effect of transgene	References
OsNAS1	*Oryza sativa*	Endosperm-specific rice glutelin and maize ubiquitin	Overexpressing *OsNAS1* in transgenic rice resulted 19%–46% and 23%–32%, respectively, higher Fe and Zn in unpolished seeds than the wild type, WT; Fe in polished seed was similar to that of WT but Zn increased by 23%–32%; overexpression of *OsNAS1* in seed showed no obvious effect on agronomic traits but more than twofold higher bioavailable Fe to human from the high nicotianamine grains than WT	Zheng et al. 2010
HvNAS1	*Hordeum vulgare*	OsActin1	Transgenic rice overexpressing *HvNAS1* showed two- to threefold increased Fe and Zn concentrations in polished T_1 seeds; increased Fe and Zn in T_2 polished and brown seeds	Masuda et al. 2009
OsNAS	*O. sativa*	CaMV 35S	Overexpression of *OsNAS* in transgenic plants resulted 2.9-fold and 2.2-fold greater seed-Fe and -Zn compared to WT, and increased tolerance to Fe and Zn deficiencies	Lee et al. 2009
AtNAS, Pvferritin	*Arabidopsis thaliana, Phaseolus vulgaris*	CaMV 35S endosperm-specific globulin	Transgenic plants containing *nicotianamine synthase* and *ferritin* genes showed more than sixfold Fe increase in rice seeds endosperm, with no yield penalty but earlier flowering	Wirth et al. 2009

Mugeneic acid (MA)	*H. vulgare*		Transgenic rice containing *IDS3* (*HvNAAT-B*) gene had up to 1.4 and 1.35 times higher seed-Fe and -Zn concentrations, respectively, compared to WT	Masuda et al. 2008
Gmferritin	*Glycine max*	Endosperm-specific glutelin	Transgenic rice cultivar, BRR 29, accumulated as much as 9.2 $mg\,kg^{-1}$ iron in the seed than the WT (3.8 $mg\,kg^{-1}$)	Khalekuzzaman et al. 2006
SoyferH-1	*G. max*	Endosperm-specific glutelin, *GluB-1*, and globulin, *Glb-1*	Transgenic rice containing double ferritin (*GluB-1/SoyferH-1* and *Glb-1/SoyferH-1*) and single ferritin (*Glb-1/SoyferH-1*) genes showed significantly higher seed-Fe (15.4–15.7 $\mu g\,g^{-1}$), about 40% higher than WT (11.2 $\mu g\,g^{-1}$)	Qu et al. 2005
Gmferritin	*G. max*	Endosperm-specific glutelin	Enhanced Fe and Zn, both in brown and polished rice, in transgenic *indica* rice IR68144	Vasconcelos et al. 2003
Pvferritin	*P. vulgaris, Aspergillus fumigatus*	Endosperm-specific glutelin	Twofold increase in Fe content of the seeds from the transgenic rice, Taipei 309	Lucca et al. 2002
Pvpfe, Afphytase, rgMT	*P. vulgaris, A. fumigatus, O. sativa*	Endosperm-specific glutelin	Transgenic rice containing *pfe* and *phytase* gene not only raised seed Fe (twofold) but also improved bioavailable Fe due to many fold increase in phytase activity which degrade phytate to release more bioavailable Fe; overexpressing *rgMT* increased the cysteine content, which further enhanced bioavailable Fe	Lucca et al. 2001
Gmferritin	*G. max*	Endosperm-specific glutelin, *GluB-1*	Transgenic rice containing *ferritin* accumulated up to threefold more seed-Fe (38.1 $\mu g\,g^{-1}$) than the WT (11.2 $\mu g\,g^{-1}$)	Goto et al. 1999

NAS gene from barley and rice increased Fe and Zn concentrations by two- to threefolds in transgenic rice compared to WT, with no obvious effect on agronomic traits (Table 3.9). Using Caco-2 cell digest model, Zheng et al. (2010) further demonstrated that elevated NA concentration led to more than twofold higher bioavailable Fe from the high NA seeds than the WT. Thus, NA is a novel and effective promoter of Fe utilization. More importantly, the transgenic rice plants containing *AtNAS* and *Pvferritin* genes increased seed-Fe concentration by sixfold, with no yield penalty but such plants were earlier to flower, and that the Fe in the endosperm of the transgenic rice lines accumulated in spots, most probably as a consequence of spatially restricted ferritin accumulation (Wirth et al. 2009). Biofortifying rice with *NAS* alone or in combination with *ferritin* has great potential in combating global human Fe deficiency in people dependent on rice for their sustenance (Lee et al. 2009; Zheng et al. 2010).

Mugineic acid family phytosiderophores (MAs) play an important role in the uptake of Fe from the soil and Fe transport within the plant in graminaceous plants. NA is the precursor of MAs that are natural Fe(III) chelators for Fe acquisition from the rhizosphere (Mihashi and Mori 1989; Takagi 1976). Rice produces DMA that chelates Fe(III) and contributes to Fe uptake and internal transport (Kobayashi and Nishizawa 2008). By hypothesizing that overexpression of the *NAS* in rice would enhance the synthesis of NA and DMA, and thus increase Fe and Zn concentrations in the seeds, Masuda et al. (2009) inserted *HvNAS1* into rice and the resultant transgenic plants showed increased *HvNAS1* expression, endogenous NA, and phytosiderophore content in shoots, roots, and seeds. They detected two- to threefold increases in Fe and Zn concentrations in transgenic polished T_1 seeds, with both the elements (NA and DMA) also high in polished and brown T_2 seeds, which clearly indicate overproduction of NA enhances the translocation of Fe and Zn into rice seeds.

2. Phytate. Alternative to mutagenesis is to either block the phytate biosynthetic pathway or degrade phytate in developing seeds to produce low-phytate crops through transgene. Phytases, known to enhance phosphate and mineral uptake in monogastric animals, catalyze the hydrolysis of phytate to *myo*-inositol pentakisphosphate (IP_5) or to less phosphorylated *myo*-inositol phosphates IP3. The phytases isolated from plant, microorganism, and animal tissues are broadly classified into three types, 3-phytases, 6-phytases, and 5-phytases, depending on the initiation site of dephosphorylation (Rao et al. 2009). A thermotolerant phytase gene from *Aspergillus* has been used to alter phytic acid in maize, rice, and soybean (Table 3.10). For example, transgenic

Table 3.10. Examples of transgenic maize, rice, and soybean with low seed phytate covering a period from 2002 to 2008.

Gene	Source	Promoter	Effect of transgene(s)	References
Maize				
phyA2	*Aspergillus niger*	Maize embryo specific globulin	A very high phytase activity (1,791–2,502 U kg^{-1} seeds) and reduced phytic acid (2.4–2.7 mμg^{-1}) in transgenic seeds compared to WT (phytase activity 38.3 U kg^{-1} and phytate content ~3.3 mg g^{-1}); stable phytase expression; no adverse effect on germination, plant growth, and seed development between transgenic and WT	Chen et al. 2008
lpa1	*Zea mays*	Ole and Glb	Multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter is highly expressed in embryos; silencing expression of this transporter produced low-phytic-acid and high-Pi (inorganic phosphorous) in transgenic maize seeds with no adverse effect on germination and plant dry weight	Shi et al. 2007
phyA	*Aspergillus*	Seed-specific rice glutelin-1	Up to 95% of the endogenous phytic acid degraded in transgenic maize seeds flour containing *phyA* gene, with corresponding increase in available phosphate	Drakakaki et al. 2005
Rice				
RINO1	*O. sativa*	Ole 18 (rice)	Phytic acid in seeds reduced by 68% in transgenic plants containing *RINO1*, with corresponding increase in available phosphate; reduced phytic acid had no adverse effects on seed weight, germination, or plant growth	Kuwano et al. 2009

(*continued*)

Table 3.10 (*Continued*)

Gene	Source	Promoter	Effect of transgene(s)	References
RINO1	*O. sativa*	Rice seed storage protein glutelin, GluB-1	Seeds of the T_5 generation of the transgenic plants containing *RINO1* had more inorganic phosphate (Pi), without a reduction in total phosphorus levels, compared to WT; increase Pi accompanied by a molar-equivalent decrease in phytic acid P	Kuwano et al. 2006
phyA	*A. fumigatus*	Endosperm-specific glutelin	Transgenic rice containing *phytase* showed enhanced Fe bioavailability	Lucca et al. 2002
Soybean				
appA	*Escherichia coli*	Soybean seed lectin	Soybean line expressing *appA* in the cytoplasm of developing cotyledons exhibited high levels of phytase expression, ≥90% reduction in seed phytic acid but concomitant increases in total free phosphate; however, it reduced emergence but had no effect on the number of seeds plant^{-1} or seed weight	Bilyeu et al. 2008
GmPhy	*G. max*	Seed-specific β-conglycinin	Phytic acid reduced by 13%–25% in transgenic seeds as compared to WT; phytase expression during seed development resulted 2.7-fold increase in available P	Chiera et al. 2004

maize containing *phyA2* gene showed a very high phytase activity (1791–2502 Unit kg^{-1} seed) and reduced phytic acid compared to WT (Chen et al. 2008).

Meanwhile, a MRP ATP-binding cassette transporter is highly expressed in embryo, and by silencing expression of this transporter, Shi et al. (2007) produced transgenic maize low in seed phytate but high in Pi. The rice Ins(3)P_1 synthase gene (*RINO1*) is highly expressed in developing seed embryos and in the aleurone layer, where phytic acid is synthesized and stored. Targeted insertion of *RINO1* in rice resulted substantial reduction in phytic acid, with corresponding increase in available Pi (Kuwano et al. 2009). In all these cases, seed-specific promoters were used and the resultant transgene(s) had no adverse effect on seed germination, plant growth and development, unlike lp mutants generated through induced mutagenesis, which are affected by poor germination and emergence as well as reduced seed weight (see Section V.A.2). The molecular approaches may be more advantageous than mutagenesis combined with traditional breeding to manipulate phytic acid biosynthesis in food crops (Kuwano et al. 2006; Blair et al. 2008).

3. β-Carotene. Transgenic approaches have been used to effectively modify the carotenoids concentration to enhance nutritional value of maize and rice (Table 3.11). For example, Ye et al. (2000) were the first to introduce the entire β-carotene biosynthetic pathway to produce transgenic rice with yellow endosperm grains containing ~1.6 $\mu g\, g^{-1}$ carotenoid, and coined the term 'Golden Rice'. Subsequent development led to creation of 'Golden Rice 2' containing exceptionally high amount of carotenoids (maximum 37 $\mu g\, g^{-1}$), of which β-carotene was the predominant (31 $\mu g\, g^{-1}$) (Paine et al. 2005). The proof of concept has been demonstrated that genes *psy* and *crtI* or *psy, crtI* and *lycopene β-cyclase* under endosperm-specific promoters have been effective for elevating β-carotene concentration in transgenic rice, with more β-carotene in polished grains. More importantly, the incorporation of genes for carotenogenesis in seeds by transgenesis or by introgression did not change any significant agronomic characteristics in rice plants (Paine et al. 2005; Baisakh et al. 2006; Datta et al. 2006, 2007).

Other researchers have used similar strategy as adapted for the creation of 'Golden Rice 2' to alter carotenoids profiles of the maize seeds (Table 3.11). Overexpression of bacterial genes *crtB* and *crtI*, under the control of endosperm-specific promoter "super γ-zein," resulted in an increase of total carotenoids of up to 34-fold with preferential accumulation of β-carotene in the maize endosperm (Aluru

Table 3.11. Examples of transgenic maize and rice with enhanced β-carotene in the seeds covering a period from 2000 to 2009.

Gene	Source	Promoter	Effect of transgene	References
Maize				
psy1	*Z. mays*	Wheat LMW glutenin	Transgenic maize containing *psy1*, *crtI*, *dhar*, and *folE* gene contains 169-fold the normal amount of β-carotene, sixfold the normal amount of ascorbate, and double the normal amount of folate, and the levels of engineered vitamins remained stable at least through to the T_3 homozygous generation	Naqvi et al. 2009b
crtI	*Pantoea ananatis*	Barley D-hordein		
dhar	*O. sativa*	Barley D-hordein		
folE	*E. coli*	Barley D-hordein		
psy1	*Z. mays*	Endosperm-specific wheat glutenin	A South African white maize, M37W, transformed with five carotenogenic genes controlled by different endosperm-specific promoters; transgenic plants expressing different enzyme combinations and showing distinct metabolic phenotypes recovered, with some accumulating maximum of 57.35 $\mu g\, g^{-1}$ β-carotene and other carotenoids but with no adverse effect on plant morphology and development	Zhu et al. 2008
crtI	*P. ananatis*	Endosperm-specific barley hordein		
Lycopene β-cyclase	*Gentiana lutea*	Endosperm-specific rice prolamin		
bch	*G. lutea*	Endosperm-specific rice glutelin-1		
crtW	*Paracoccus*	Endosperm-specific maize γ-zein		

crtB and *crtI*	*Erwinia herbicola*	Super γ-zein	Overexpression of *crtB* and *crtI* resulted in an increase of total carotenoids of up to 34-fold with a preferential accumulation of β-carotene in maize endosperm, which remained reproducible over at least four generations	Aluru et al. 2008
Rice				
psy	*Narcissus pseudonarcissus*	Endosperm specific rice glutelin	Polished seeds of transgenic rice showed the presence of higher accumulation of β-carotene, Fe and Zn compared to WT; prussian blue staining revealed the presence of Fe in the endosperm cells of transgenic rice seeds compared to WT where Fe restricted only to aleurone and embryo	Sellappan et al. 2009
crtI *psy*	*Erwinia uredovora* *N. pseudonarcissus*	CaMV 35S Endosperm-specific glutelin	BR29 rice containing *psy* and *crtI* showed up to 9.34 $\mu g\, g^{-1}$ total carotenoids, with β-carotene approaching 3.92 $\mu g\, g^{-1}$ in polished seeds; the total carotenoids and β-carotene in polished seeds of transgenic rice IR64, respectively, 2.32 and 0.96 $\mu g\, g^{-1}$	Datta et al. 2006
crtI *psy*	*E. uredovora* *N. pseudonarcissus*	CaMV 35S Endosperm-specific rice glutelin	Golden Rice 2 containing *psy* and *crtI* showed a total carotenoids of up to 23-fold (maximum 37 $\mu g\, g^{-1}$), with a preferential accumulation of β-carotene (31 $\mu g\, g^{-1}$), compared to the original Golden Rice (1.6 $\mu g\, g^{-1}$) (Ye et al. 2000); transgene had no adverse effect on plant phenotype, seed weight, or germination	Paine et al. 2005

(*continued*)

Table 3.11 (*Continued*)

Gene	Source	Promoter	Effect of transgene	References
psy	*N. pseudonarcissus*	Endosperm-specific rice glutelin	Total carotenoids in transgenic *indica* rice cultivars ranged from 0.297 to 1.05 $\mu g\,g^{-1}$, with stable trait expression over two generations; transgenic with only *psy* and *crtI* had same amount of β-carotene as reported in original Golden Rice (Ye et al. 2000)	Datta et al. 2003
crtI	*E. uredovora*	CaMV 35S P		
Lycopene β-cyclase	*N. pseudonarcissus*	CaMV 35S P		
psy	*N. pseudonarcissus*	Endosperm-specific rice glutelin	Using *Agrobacterium*-mediated transformation, the entire β-carotene biosynthetic pathway introduced into rice endosperm into single transformation effort with three vectors; the transformed endosperm were yellow, with pB19hpc single transformants showing a 3:1 segregation (colored vs. noncolored endosperm) whereas the pZPsC/ZLcyH cotransformants showed variable segregation; and the endosperm of a single transgenic line, z11b, had 1.6 $\mu g\,g^{-1}$ carotenoid	Ye et al. 2000
crtI	*E. uredovora*	CaMV 35S		
Lycopene β-cyclase	*N. pseudonarcissus*	Endosperm-specific rice glutelin		
aphIV		CaMV 35S		

et al. 2008), which remained stable over four generations and that the increased accumulation of β-carotene is due to an up-regulation of the endogenous lycopene β-cyclase.

Recent developments in the genomic revolution provide researchers a great deal of information, which can be derived from studying many genes or proteins simultaneously using multigene transfer (MGT) as an approach to generate plants with more ambitious phenotypes, for example, the import of entire metabolic pathways, the expression of entire protein complexes, the development of transgenic crops simultaneously engineered to produce a spectrum of added-value compounds, with limitless potential (Naqvi et al. 2009a). This approach has been recently employed to generate transgenic maize containing four to five genes to enhance the nutritional profiles of the maize kernels (Table 3.11). For example, transgenic maize containing *psy1*, *crtI*, *dhar*, and *folE* genes under endosperm-specific promoters contains 169-fold the normal amount of β-carotene, sixfold the normal amount of ascorbate, and double the normal amount of folate (Naqvi et al. 2009b). Zhu et al. (2008) introduced five carotenoid genes, *psy1*, *crtI*, *lycopene β-cyclase*, *bch*, and *crtW* in a South African white maize cultivar 'M37W'. The transgenic events express different enzyme combinations to show distinct metabolic profiles, with some accumulating up to 57.35 $\mu g\, g^{-1}$ β-carotene and other carotenoids but with no adverse effect on plant morphology and development, which has allowed them to identify and complement rate-limiting steps in the pathway and to demonstrate competition between β-carotene hydroxylase and bacterial β-carotene ketolase in four sequential steps of the extended pathway.

Thus, the combinatorial transformation is a versatile approach that could be used to modify any metabolic pathway and pathways controlling other biochemical, physiological, or developmental processes. These examples clearly demonstrate that through use of transgenic technology, it is possible to alter the seed composition to make food crops more nutritious to human health. However, use of transgene still has some practical limitations, for example, mechanism and pattern of gene integration, dosage effect due to variable copy insertion, interaction between transgenes, rearrangements and silencing, promoter choice as a function of gene number (i.e., one promoter vs. more promoters when integrating multiple genes) as repetitive promoters may in some cases have negative impact on transgene stability and expression, or proper coordination of all enzymes involved in the metabolic pathway. Transfer of an incomplete pathway induces significant changes in plant morphology, variable expression of transgene effects in different generation and some lines with more expression than

others in later generations etc (Kristensen et al. 2005; Naqvi et al. 2009a,b; Dietz-Pfeilstetter 2010; Peremarti et al. 2010).

It is, therefore, evident that the best approach to address human nutrition through biofortification strategies will likely involve genetic engineering in conjunction with conventional breeding, particularly when the direct enhancement of local elite breeding is required (Datta et al. 2007; Naqvi et al. 2009a,b; Ronald 2011).

B. Consumer's Attitude to Genetically Modified Biofortified Crops

Biofortification often alters the flavor, taste, appearance (not in the case of Fe/Zn), and other features of foods that may limit consumer acceptance of the nutritionally enhanced GM food. It is, therefore, worth investigating the social and economic impacts of nutritionally enhanced transgenic plants such as the cost-effectiveness of adapting local cultivars, social acceptance of the strategy, willingness to pay for genetically modified food (Kimenju and Groote 2008; Stevens and Winter-Nelson 2008; González et al. 2009), and the overarching regulatory policy (see Section VII.C) for producing such crops on an agronomic scale (Ramessar et al. 2008). The proof of concept of developing nutritionally enhanced biofortified crops through genetic engineering have been demonstrated in maize and rice (see Section VII.A). The overexpression of genes associated with increased Fe concentration did not change the seed color in rice, while enhanced β-carotene resulted golden-yellow seeds in rice, orange-yellow seeds in maize, and orange colored cassava and sweet potato. Skepticism prevails among public in large about the acceptance of, for example, β-carotene-enriched rice (golden rice), cassava, and sweet potato. The consumers in southern Africa have strong preference for white maize (which has no β-carotene) and often consider yellow maize being inferior, suitable only for animal feed (Rubey et al. 1997). How far the biofortified β-carotene-enriched maize will be accepted by the consumers who prefer white maize for consumption is a question remaining to be answered. A survey conducted in Kenya reveals that consumer preference is influenced by socioeconomic factors such as gender, education, and income or ethnic background. For example, in spite of the strong preference for white maize, many would prefer to eat yellow (biofortified) maize if offered a price discount (~37%) over white maize. Women being more sensitive to nutrition have shown preference to eat biofortified maize than men, and urban consumers (due to raise in income and more awareness about the micronutrient malnutrition) have shown a willingness to pay for biofortified maize. Consumers from western Kenya have a lower

preference for white maize, while those from central Kenya a stronger preference for biofortified yellow maize (Groote and Kimenju 2008). The survey results also reveal that biofortified β-carotene-enriched maize and OFSP in Mozambique and cassava in northeast Brazil is acceptable to many consumers in these countries (Low et al. 2007; Stevens and Winter-Nelson 2008; González et al. 2009). The yellow gari (made from biofortified cassava containing high β-carotene) in Nigeria is fetching a higher price than white gari (HPlus Nigeria Project). Furthermore, in a recent study on acceptance of biofortified sweet potato in Uganda, Chowdhury et al. (2011) found that taste plays an important role in consumer acceptance; however, the provision of nutrition information does translate into substantial premiums for the biofortified sweet potato relative to the more common white cultivars. The consumers in Uganda are willing to pay for biofortified cultivars as much they are for the currently consumed traditional (white) cultivars.

The adoption of nutritionally enhanced food crops will improve the health and well-being of the world's poorest people, but this advancement will only be possible if political differences over the development and use of transgenic crops are set aside and their deployment and cultivation is regulated according to robust, science-based criteria (Naqvi et al. 2009a,b; Ramessar et al. 2009; Gómez-Galera et al. 2010).

C. Nutritionally Enhanced Genetically Modified Crops and Biosafety Issues

The GM crops are currently classified in generations and according to the objective of the trait being introduced. The first generation GM crops include those that possess resistance to herbicide and/or insect pests (input trait), while the second generation GM crops include those with new traits of direct value to consumers such as improved grain quality (output trait). The third generation of GM crops being developed is expected to confer plants a greater ability to resist abiotic stresses (drought, salinity and high temperatures), provide additional health benefit or renewable raw materials (bioenergy-rich crops) or pharma-plants to produce high-grade active pharmaceutical ingredients (Magaña-Gómez and de la Barca 2008). The first generation GM crops are the herbicide-resistant soybean, insect-resistant maize and cotton, herbicide- and insect-resistant potato; the second generation GM crops are rice with enhanced β-carotene and/or higher iron and zinc levels or maize with high phytase, while maize with enhanced multiple vitamins and minerals could be classified as third generation crop (see Section VII.A). The herbicide- and/or insect pest-resistant soybean,

maize, and cotton are commercially grown in 125 million ha in 25 countries (James 2008), while nutritionally enhanced GM crops are yet to be commercialized. The future waits for the success or limitations of second and third generation transgenics.

The major concern about the GM crops/foods is the safety to humans and animals. The potential risks to human health include toxicity, allergenicity, the instability of inserted gene, and negative effects on nutrition (Conner and Jacobs 2000). During 1996–2010, a large number of studies on the safety assessment of GM crops (maize, peas, potato, rice, soybean, sweet pepper, and tomato) or GM traits (herbicide, insect, and virus resistance) have been conducted using various feeding periods, animal models (broiler, catfish, chicken, cow, dairy cattle, mouse, mouse testes, rabbit, rat, salmon, sheep, and steer), and parameters (body weight, feed consumption, blood chemistry, organ weight, and histopathology). The most common findings from these investigations have varied from no alteration of the nutritional value of the GM food tested to minimal detrimental effects on the nutritional value to *in vivo* submicroscopic effects in different animal species.

Differences among the methods employed for evaluation, and the varied results obtained on the risk assessments reflect the complexity of the problem and therefore, there is an urgent need to harmonize the methods used to evaluate the safety of the GM foods (Flachowsky et al. 2007; Magaña-Gómez and de la Barca 2008; Magaña-Gómez et al. 2008; de Vendômois et al. 2009; Haryu et al. 2009; Steinke et al. 2010). Rigorous, multigenerational animal safety assessments should be done to identify the risks to health, and all the GM products including those nutritionally enhanced by biofortification must be labeled to be monitored for the long-term adverse health consequences due to their consumption.

The detection and characterization of the unintended effects of the genetic modification, as demonstrated in maize (higher lignin content in Bt maize by Saxena and Stotzky 2001) and soybean (depleted plant flavonoids in herbicide-tolerant soybeans by Lappé et al. 1999), continues to be an issue that needs further investigation. The newly developed methods of screening for potential alterations in the metabolism of the modified organism include the analysis of gene expression (microarrays, mRNA finger printing), overall protein analysis (proteomics), and secondary metabolites (HT-UHPLS-TOF-MS, MRM-TQMS; Grata et al. 2009; Sawada et al. 2009; Allwood and Goodacre 2010) that should be integrated into the risk assessment process. To sum up, the advances made in molecular biology, toxicology, biochemistry, and

nutrition hold the promise of providing sets of genes and methodologies that serve as biomarkers for a cell's responses to toxins, allergens, or other compounds, which hold potential to the development of new tools to assess the GM crops. Further, the next step for a safe use of the GM crops is to adopt strictly the recommendations made available by the regulatory agencies to ensure that the consumption of GM foods does not pose a serious health hazard to humans, animals, and biota (Magaña-Gómez and de la Barca 2008).

It is encouraging to note that a number of countries have put in place the laws and biosafety regulations governing the tolerance levels for the GM materials in nonGM food and in the labeling and traceability of the GM products. However, in many developing countries, there appears to be no tolerance limit to distinguish the GM and nonGM food and feed. Furthermore, there is no uniformity in the approach to adopting the labeling and traceability of the GM food among the countries, that is, while some adopted voluntary leveling of the GM products, others adopted mandatory labeling of GM products, still others have no requirements for labeling at all, which is a serious problem when such GM crops/products are traded for food and feed uses, therefore an urgent need exists to harmonize the regulations on a global level (Ramessar et al. 2008).

The environmental risks associated with the GM crops such as gene flow, adverse effects on biodiversity and on the beneficial insects, and the potential emergence of superweeds are not referred to here as these issues have been adequately dealt elsewhere (Wolfenbarger and Phifer 2000; Andow and Zwahlen 2006; Kwaku and Asante 2008; Dunfield and Germida 2010; Hokanson et al. 2010; Jiang et al. 2010; Liu 2010; Raybould et al. 2010).

In summary, the *ferritin* gene either from soybean or common bean or *NAS* gene from *Arabidopsis* driven by endosperm-specific promoters have been used to enhance seed-Fe concentration. The transformed rice containing *AtNAS* and *Pvferritin* has increased iron several fold compared to WTs with no yield penalty. Phytate limits the Fe bioavailability. The transgenic rice containing *RINO1* shows substantial reduction in phytic acid, but with corresponding increase in available inorganic phosphorus, and the produce from such plants when sown had no adverse effects on seed germination, plant growth, and development, unlike lp mutants. The transgenic maize containing *phyA2* from *Aspergillus* shows high phytase activity and reduces phytic acid compared to WT. A new version of Golden Rice named as 'Golden Rice 2' has been developed, which has exceptionally high amount of β-carotene (31 $\mu g\, g^{-1}$ seed), which is available to the public domain for large-scale evaluation

and introgression into locally adapted rice cultivars. Multigene transfer is an approach to generate plants with more ambitious phenotypes. Transgenic maize containing five carotenoid genes show higher accumulation of β-carotene with no adverse effects on plant morphology and development. It has been clearly demonstrated that it is possible to alter the seed composition, using transgene(s), to make food crops more nutritious to human health; however, the use of transgene still has some practical limitations, highlighted in this section, which must be addressed to minimize failures and disappointments.

Biofortification has the potential to alter the flavor, taste, or appearance, which may limit consumer acceptance. The enhanced β-carotene results in golden-yellow colored seeds in rice, the orange-yellow seeds in maize, and orange in cassava and sweet potato. The limited survey of β-carotene-enriched maize or orange-fleshed sweet potato and cassava in some countries in Africa and northeast of Brazil reveals that such products are acceptable to many consumers. More such studies are needed to gauge consumer's preference to biofortified crops. The deployment and cultivation of nutritionally enhanced GM crops will succeed only if the political differences over the development and use of GM crops are set aside and a rigorous assessment is in place based on robust, science-based criteria to assess biosafety issues associated with the use of GM crops/products.

VIII. OUTLOOK

Limited studies involving germplasm have shown potential variability that merits further exploration to mine genetic variation for grain Fe, Zn, phytate, and β-carotene in germplasm collections. The core or mini-core collections available in most of the cereal and legume crops should form the basis to identify seed mineral-dense germplasm. Priority may be given to screen germplasm from regions with soils deficient in micronutrients as the germplasm from such regions generally develops inherent adaptation mechanisms, favoring enhanced nutrient uptake, transport, distribution, and relocation in plants/seeds. Wild relatives are another valuable resource to explore variation for micronutrients, as evidenced in common bean and wheat. Although few major QTL associated with increased seed-Fe/-Zn concentrations and/or Fe bioabailability have been reported in common bean, maize, rice, and wheat; more such studies are needed to understand the genetics of seed micronutrients concentration and bioavailability. The response of cultivars to the production

environments need to be characterized to further improve the probability of predicting and identifying seed mineral-dense germplasm/cultivars. A useful strategy would be to characterize the production environment prior to evaluating germplasm and breeding lines in the target environments for identifying seed mineral-dense lines adapted to such production environments. The bioavailability of micronutrients is an issue that should be factored in when selecting for seed mineral-dense cultivars; however, such studies are costly and time consuming. The *in vitro* Caco-2 cell assay should be used as a first step to assess the bioavailability, while the *in vivo* assay may be limited only to potential cultivars/hybrids to complement the *in vitro* results, prior to their release for commercial cultivation.

Nutrient acquisition in plants is largely influenced by root architecture, root exudates, and mycorrhizal associations. How nutrients from the soils are absorbed, transported, and partitioned into various plant parts and localized in seeds merit further investigation to develop selection criteria for the development of seed mineral-dense cultivars. High nutrient-use efficiency, as measured by high harvest index for micronutrients (HI_{Fe} or HI_{Zn}) should be integrated in breeding programs together with high $HI_{seed\ yield}$.

High-throughput assays are needed to facilitate large-scale screening of seed samples for micronutrient concentration. XRF has emerged as a rapid and cost-effective assay for initial screening to discard lines in the lower range of Fe and Zn. Subsequently, promising lines must be analyzed for variation by ICP or AAS methods. The accumulation and spatial distribution of macro- and micronutrients provide elemental maps in whole seed, and the information from such distribution pattern should be integrated into the selection strategies for the biofortification of staple crops. Such information is also warranted to minimize micronutrient losses during the milling/polishing processes.

The proof of concept has already been demonstrated that it is possible to develop seed micronutrient-dense biofortified crops by exploiting natural genetic variation with no yield penalty, as evidenced in maize, cassava, and sweet potato (β-carotene), common bean, pearl millet, and rice (iron). Seed iron-dense common bean and rice in several countries in Latin America; seed iron-dense common bean in some countries in Africa; and seed iron-dense rice in the Philippines in Asia have been released for cultivation. The β-carotene biofortified maize in Kenya, Mozambique, Nigeria, and South Africa, and β-carotene biofortified sweet potato in Mozambique and Uganda and cassava in Nigeria and DR Congo, all derived by using naturally occurring allelic variation, are being assessed for adaptation, acceptability, and efficacy prior to release

in these countries. A paradigm shift is needed to include biofortification in core breeding programs to assure that no crop cultivars that do not meet the minimum seed-micronutrient density are released for cultivation.

The use of transgenic approaches to enhance the nutritional quality of food crops have been demonstrated in rice (β-carotene and iron) and maize (β-carotene). Genetic variants of 'Golden Rice 2' are being introgressed to transfer high β-carotene trait into several Asian rice cultivars, which will soon be available in public domain (Barry 2011). Reducing phytic acid enhances the bioavailability of Fe/Zn, which could be achieved either by blocking the phytate biosynthetic pathway or degrading phytate in the developing seeds. Transgenic maize containing *phyA2* or transgenic rice containing *RINO1* showed substantial reduction in phytic acid, with no adverse effects on seed germination, plant growth, and development, unlike in lp mutants. Further, MGT through transgenic approach has shown several fold improvement in the nutritional profiles of maize kernels, suggesting that MGT is a viable option to import entire metabolic pathways with limitless potential to alter the seed composition and make food crops more nutritious for human health. However, it is important to address the environmental and biosafety issues associated with the use of transgenic crops.

The critical issue to investigate is (1) whether heterosis can be exploited to enhance seed Fe and Zn; (2) whether simultaneous selection can be practiced to enhance per se the micronutrients (Fe and Zn) and their bioavailability; (3) how much low phytate per se can be reduced that is not detrimental to plants and the produce is beneficial to human health; (4) whether low-phytate trait can be combined with increased grain Fe and Zn concentrations; and (5) whether Fe/Zn (invisible trait) can be combined with marker (such as grain color) to differentiate between biofortified and nonbiofortified produce in the market. The equally important issues with respect to provitamin A research include (1) whether heterosis can be exploited to enhance β-carotene, (2) whether allelic variation for additional genes in carotenoids biosynthetic pathway be identified to reduce postharvest/storage losses, (3) whether enhancers of β-carotene bioavailability be identified and selected in breeding, (4) whether a rapid and cost-effective high-throughput assay be developed to enhance breeding efficiency, and (5) whether carotenoids have mycotoxin-reducing effects in grain (Pixley et al. 2011b). More studies are needed to assess the efficacy of biofortified products in raising micronutrient status in human subjects. Biofortification often alters the flavor, taste, appearance, and other features of the foods, which may limit the consumer acceptance–an issue that merits further investigation. To address these

and other issues, a network of global interdisciplinary partnership is suggested with a range of stakeholders, including those involved in breeding, molecular biology, food technology, human nutrition, economics, seed systems, farm extension, food product development, marketing, and public awareness to strengthen crop biofortification programs both in the developed and developing countries (Bouis and Islam 2011). The biofortified crops must be adopted by farmers and consumed by those suffering from micronutrient malnutrition. To make this happen, it is suggested that the biofortified produce should be made available to public through the public food distribution system (PFDS), which can create an institutional demand for biofortification and will surely work if high volumes of biofortified crops are procured through the PFDS, targeting the poor. The PFDS has the potential to induce farmers to cultivate biofortified crops by providing price support for production. Moreover, for enhancing the integration of biofortification into the PFDS, it is important that policymakers are made aware of the benefits of biofortification (Ahmed 2011).

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