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Biofortification of Staple Crops

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Foreword

The United Nations' Sustainable Development Goals (SDGs) target to end poverty, to protect the planet, and to ensure that all people enjoy peace and prosperity by 2030. The overarching challenge for the CGIAR scientific community is how to support countries to achieve SDGs by ensuring food and nutrition security to an ever-increasing population from limited and fast depleting resources under a climate crisis. Malnutrition is a serious global burden with ~800 million people being undernourished, over 2 billion suffering from micronutrient deficiencies, and more than a third of the adult population obese or overweight. Estimates indicate that over 60% of the world's 7 billion people are iron (Fe) deficient, over 30% are zinc (Zn) deficient, 30% are iodine (I) deficient, and more than 15% are selenium (Se) deficient, often causing health problems and development delays in those suffering from these deficiencies in spite of the fact that the global food production has increased manifolds. Failure to link agricultural production with human nutrition and health has led to the development of unhealthy food systems. Biofortified crops, which have been bred to have higher amounts of micronutrients, can help provide these essential vitamins and minerals. They are effective in reducing hidden hunger caused by micronutrient deficiencies and are an integral component of food-based approaches to improve nutrition and food security.

Realizing the importance of micronutrients in human diets and their role in waning the hidden hunger among the poor masses, scientific community has recently placed major emphasis on biofortification of staple crops to augment the micronutrient availability with no cost at consumer end. Since 2003, the HarvestPlus program of CGIAR has added nutritional value into staple crops to address micronutrient deficiency among smallholder farming families and other low-resource populations. More than 50 million people in smallholder farming families in 41 countries now benefit from biofortified crops, which are making a measurable impact on human nutrition, health, and development. Presently, biofortified crops, including vitamin A orange sweet potato, iron beans, iron pearl millet, vitamin A yellow cassava, vitamin A orange maize, zinc rice, zinc wheat, and iron-rich lentils have been released in more than 30 countries. The technological advancement during the process has led to a great volume of research on trait discovery and deployment related to micronutrients in stable crops, and their bioavailability and efficacy in human health. Such efforts will further be augmented in the One CGIAR 2030 Research and

Innovation Strategy for achieving the SDGs by transforming food, land, and water systems under the genetic innovation to develop varieties with higher levels of vitamins and minerals that are adapted to a wide range of agro-ecological conditions and ensuring that the best germplasm for climate-adaptive and consumer-preferred traits continues to be used in breeding biofortified crops.

While the genetic diversity for micronutrient content in the existing germplasm is the basic need for mainstreaming biofortification in crop improvement program, community access to comprehensive information is key to further scientific efforts for developing nutrient-rich cultivars towards strengthening human health and nutrition efforts. However, information generated on various aspects of biofortification is scattered in different journals, and the researchers and scholars spend considerable time and energy in searching the relevant literature for their research and study. The present book '**Biofortification of Staple Crops**', which is a meticulously edited volume, is an attempt in this direction to bring together information on various aspects of biofortification and agronomic interventions. Twenty chapters in the book have been contributed by the renowned scientists whose research contributions on biofortification are acknowledged globally. I am quite hopeful that the information contained in this book will boost research efforts of plant scientists to bring about a major breakthrough in biofortification and will serve as a resource material for those who are involved in teaching, in research, and in technology scaling in agricultural crops. I congratulate the editors Drs. **Shiv Kumar**, **Harsh Kumar Dikshit**, **Gyan Prakash Mishra**, and **Akanksha Singh** for bringing out this book timely on such an important and emerging aspect and hope that it would be widely read by scholars and researchers.

ICARDA
Cairo, Egypt
February 22, 2021

Jacques Wery

Preface

Micronutrient deficiency is a leading global concern of public health importance. The root cause of this problem is non-availability of balanced diet to resource-poor communities. Resource poor rely on staple food for their energy requirement and these staple food crops are low in micronutrient concentration. Therefore, the biofortification of staple crops is essential to restrict malnutrition and diseases and promoting well-being of target population. Among the micronutrients, vitamin A, iron, and zinc are the most common deficiencies reported from economically disadvantaged communities posing detrimental effect on health and well-being of affected communities. Preschool children below the age of 5 years and women of reproductive age are most affected by micronutrient deficiencies.

Globally 50% deaths of under-5 years are associated with vitamin A, zinc, and iron deficiency. Vitamin A deficiency causes night blindness, child morbidity, and mortality. Iron deficiency causes anaemia, maternal and childhood deaths, and poor cognitive development. Nearly 60% global population suffers from iron deficiency. Zinc deficiency causes reduction in linear growth, diarrhoea, and impaired immunity among 30% of global population. The acute deficiency of vitamin A, Fe, and Zn causes childhood stunting. The stunted children exhibit poor cognitive development and have risk of developing cardiovascular diseases, obesity, and type 2 diabetes. It is estimated that globally the decline in productivity due to loss of cognitive skills, stunting, and chronic diseases is likely to cost $35 trillion by 2030.

The United Nation's Sustainable Development Goals (SDGs) SDG 2 and SDG 3 focus on eradication of micronutrient deficiencies. National Research Programmes are joining hands with CGIAR institutes to achieve SDGs ensuring food and nutritional security for all. Biofortified crops with relatively higher micronutrient concentration are sustainable means to address micronutrient deficiency for resource-poor communities. The limited efficacy studies conducted have indicated their role in improving micronutrient availability. The joint efforts of HarvestPlus programme and National Research Programmes have led to development and dissemination of biofortified varieties of different crops in different countries. Biofortified crops have been developed in wheat, rice, maize, pearl millet, cassava, sugar beet, dry beans, lentil, and several other crops benefitting nearly 50 million people in small farming families.

The current book "**Biofortification of Staple Crops**" compiles research and technological advances made in evaluation of genetic resources, gene discovery, product development, bioavailability, and efficacy studies for different crops. The twenty chapters included have been compiled by leading scientists working on biofortification of respective crops. The editors are hopeful that compiled information will serve as basic resource material for teachers, students, and researchers and proliferate the productivity and impact of biofortification.

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The editorial team expresses sincere thanks to all the contributors for their valuable contribution, dedication, patience, and efficiency. Editing a multi-author book is a tedious task. However, in this case it was encouraging and a learning experience. All authors and co-authors responded promptly with consequence that the manuscripts were delivered on time without any difficulty. This helped editors in preparing the final text for publisher timely.

Editors convey deep gratitude to all who have rendered invaluable assistance in making this publication possible. Authors express their gratitude to Dr. Jacques Wery, Deputy Director General—Research, ICARDA, and Dr. A.K. Singh, Director, ICAR-IARI, for all support for completion of this book. Lastly, we thank Springer Nature for publishing this book.

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About the Editors

Shiv Kumar leads ICARDA's Food Legumes Program delivering improved germplasm of lentil, kabuli chickpea, faba bean, and grass pea to national partners in South Asia, Sub-Saharan Africa, West Asia, and North Africa. He works on developing short-duration, climate-smart varieties of lentil and grass pea, with high iron and zinc content for the sustainable intensification of cereal-based cropping systems. During his three-decade career, he has worked with national and international partners to develop 39 lentil, 5 mung bean, 2 urdbean, 1 rice, and 3 grass pea varieties. He has published 196 peer-reviewed journal articles, 77 book chapters, 10 books, 7 technical bulletins, and 2 training manuals. He also supervises teaching and training of national partners. He has done his master's and PhD in plant breeding from G.B.P.U.A. & T., Pantnagar (India).

Harsh Kumar Dikshit is serving as principal scientist at ICAR-Indian Agricultural Research Institute, New Delhi (India). He is working on genetic improvement of grain legumes through conventional and molecular approaches. He has developed 15 varieties of different grain legumes (lentil, mung bean, and dry beans) for cultivation in India. His present focus is on basic and applied research on biofortification and biotic stresses of lentil and mung bean. He is faculty of Division of Genetics and is involved in teaching of different courses and thesis research guidance to postgraduate students. He has done his master's and PhD in plant breeding from G.B.P.U.A. & T., Pantnagar (India).

Gyan Prakash Mishra is currently working as principal scientist at ICAR-Indian Agricultural Research Institute, New Delhi (India). Before that he has worked at DRDO-Defence Institute of High Altitude Research, Leh (India), as Scientist 'C' and at ICAR-Directorate of Groundnut Research, Junagadh (India), and ICAR-Indian Institute of Vegetable Research, Varanasi (India), as senior scientist. His major research interest includes crop improvement through conventional, molecular, and transgenics approaches. He has done his master's and PhD in genetics from Indian Agricultural Research Institute, New Delhi (India), and postdoc from University of California, Riverside, and Purdue University, USA, as BOYSCAST-Fellow.

Akanksha Singh is working as assistant professor at Amity Institute of Organic Agriculture, Amity University, Noida (India). Her research areas include characterization of Lens species for grain Fe and Zn and association mapping and development of genetic and genomic resources. She has published around 23 research papers in reputed journals. She has contributed to the development of lentil varieties for central India and registration of biofortified lentil germplasm L 4704 (Fe and Zn rich). She received Babu Jag Jivan Ram Memorial Gold Medal and Chancellor's Silver Medal from Bundelkhand University, Jhansi, for her master's degree. She has done PhD in biotechnology from Banasthali University, Banasthali (India).

Biofortification of Staple Crops: Present Status and Future Strategies

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Shiv Kumar, Harsh Kumar Dikshit, Gyan P. Mishra, Akanksha Singh, M. Aski, and P. S. Virk

Abstract

Micronutrient deficiencies affect nearly one-third of global population. Biofortification of staple crops is considered as a long-term and sustainable approach to ameliorate micronutrient deficiencies The review summarizes the need for biofortification, conventional breeding, genetic variation for micronutrient concentration of different crops, quantitative trait loci identified in different crops for micronutrient concentration, transgenic approach, status of release of biofortified crop varieties and efficacy of biofortified crop varieties. Research efforts focus on increasing both micronutrient concentration and bioavailability. Key challenges (mainstreaming biofortification, building consumer demand and integration of biofortification in policies, programs and investments) have been briefly highlighted. The achievements made in the biofortification of staple crops are very promising and raise hope for nutritional security for all.

Keywords

Biofortification strategies · Biofortification · Micronutrient bioavailability · Staple crop biofortification · Biofortification status

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1 Introduction

The global population is expected to reach 9.8 billion by 2050 (https://www.un.org/development/desa/en/news/population/world-population-prospects-2019.html). High yielding varieties with better nutritive value are required to meet the nutritional demands of this population. Nutritive diet is not affordable and accessible to all and 820 million people face food shortage with undernutrition affecting 10.8% global population (FAO, IFAD, UNICEF, WFP and WHO 2019). The term micronutrient includes vitamins and micronutrients required from diet for maintenance of normal molecular and cellular function. Micronutrient deficiencies (MND) are a major concern in developing countries with poor access to healthcare due to a lack of resources and medical staff. Preschool children below the age of 5 years and women of reproductive age are most affected by MND (Nestel et al. 2006; Bailey et al. 2015). MND results in poor health reducing educational attainments, work capacity and earnings (Bailey et al. 2015). MNDs affecting individuals have an adverse effect on human capital and economic development of the country. Iron is part of haemoglobin and myoglobin and is involved in transport and storage of oxygen. Iron is a component of electron transport particles, ribonucleotide reductase enzyme system and functions as a catalyst in the production of free radicals.

Iron deficiency is a common cause of anaemia. It affects 43% of preschool children and 38% of pregnant women (WHO 2015). During pregnancy, iron is required for expansion of erythrocyte mass, plasma volume and foetal placenta growth (Scholl 2005). More than 2 billion people are anaemic due to Fe deficiency. Iron deficiency during pregnancy results in maternal anaemia and reduced new borne iron store. Iron deficiency can result in impairment of cognitive function, growth retardation and low productivity. The assessment of iron status is based on plasma ferritin concentrations at $\geq$30 μg/L (iron sufficiency), 15 to $<$30 μg/L (modest iron depletion) and $<$15 μg/L (severe iron depletion) (Loy et al. 2019).

Zinc is an important micronutrient playing a critical role in gene expression and cell development and division (Hambridge 2000). Zinc is reported from all fluids and body tissues. Zinc is a component of 300 enzymes regulating protein, nucleic acid lipid and carbohydrate synthesis and degradation. Zinc maintains cell and organ integrity by stabilizing cellular membrane and components. Zinc plays a key role in polynucleotide transcription. Zinc content of average human body is estimated as 30 mmol (2 g). Zinc is vital for immune system as it affects cellular and humoral immune response (Hojyo and Fukada 2016). Stunting is very common in preschool children. Stunting affects more than 250,000 preschool children. Zinc deficiency can contribute to stunting, as it restricts growth and decreases resistance to infections (Prasad 2013). Zinc deficiency results in growth retardation, diarrhoea, delayed sexual and bone maturation, skin lesions and impaired appetite (Hambridge 1987).

Selenium is associated with thyroid hormone metabolism, antioxidant defence system and oxidative metabolism and immune system (Fairweather-Tait et al. 2011). Selenium deficiency causes thyroid dysfunction, cardiovascular disasters, the spread of viruses or tumour disorders (Tamas 2000) and reduces sperm viability (Rayman 2002). The studies of Rayman (2012) have associated Se deficiency with Down's

syndrome and congenital hypothyroidism. Endemic cardiomyopathy (Keshan disease) was recorded in Se-deficient regions in Keshan County, China. Keshan disease is a heart disorder connected with cardiogenic shock and/or congestive heart failure. Another disease reported from Se-deficient regions in the world is Kashin Beck disease (degenerative osteoarthropathy).

Vitamin A is crucial for visual function, tissue differentiation, organogenesis and immune response (Sommers 1995). Vitamin A deficiency causes night blindness and increases the risk of disease and mortality from infections (WHO 2014; Stevens et al. 2015). Vitamin A deficiency may also occur due to malabsorption and liver diseases (Rosen et al. 2015). Serum retinol below 0.70 μM/L indicates vitamin A deficiency. Early symptoms of vitamin A (hemeralopia and xerophthalmia) are often overlooked. Severe deficiency results in impairment of mucosae, sensory organs, bone marrow, skin endocrine and immune systems (Balint 1998). Severe prevalence of vitamin A deficiency among children of 6–59 months in Asia and Africa has been reviewed by Stevens et al. (2015).

Iodine is constituent of thyroxine (T4), triiodothyronine (T3) and thyroid hormones and is required for development, growth and metabolism from pregnancy, infancy to throughout life (FAO/WHO 2005; WHO/UNICEF/ICCIDD 2008). Iodine deficiency during pregnancy and childhood can cause mental retardation due to impaired growth and brain development. Zimmermann and Andersson (2012) reported iodine deficiency in 29.8% of school children. Iodine deficiency causes cretinism and goitre, mental retardation, hypothyroidism, prenatal death, infant mortality and decreased fertility (WHO/UNICEF/ICCIDD 2008). Folates are necessary for methylation cycle and biosynthesis of pyrimidines and purines (Scott et al. 2000). Folate deficiency results in the reduction of capacity to synthesize DNA and rate of cell division. Anaemia is caused due to reduction in biosynthesis of cells in the bone marrow. Neural tube defects have been reported in children of women deficient in folates in first 28 days after conception (March of Dimes 2006).

Dietary diversification, supplementation, fortification and biofortification are being used for reducing the MNDs. Biofortification is the process of increasing vitamin and mineral density in a crop through conventional plant breeding, transgenic approach or agronomic practices. The regular consumption of staple biofortified crops produces measurable improvement in human nutrition and health. The main reason for micronutrient deficiencies in developing countries is poverty. Due to lack of resources people rely on staple crops to meet their energy requirements. Animal products, protein rich food, vegetables and fruits are not part of regular diet. Areas low in soil bioavailable micronutrients produce grains low in micronutrients (Vanlauwe et al. 2015).

The plasma and tissue micronutrient concentration reflects the dietary intake. Inflammation and infection can alter the partitioning of micronutrients in the body (Thurnham and Northrop-Clewes 2016). To identify infection, C-creative protein (CRP) and α 1-acid glycoprotein (AGP) tests (Thurnham et al. 2015) are routinely conducted following the methodology appropriate for each micronutrient. Intestinal infection caused by helmithosporium affects nutritional status by reducing micronutrient absorption and increasing anaemia risk due to worm feeding on blood and

causing loss of appetite (Chaparro and Suchdev 2019). Supplementation is another strategy to address MND. Vitamin A and zinc supplementation have been successful (Black et al. 2008). Global initiative on vitamin A has prevented 1.25 million deaths (WHO 2018). Supplementation requires access to medical facilities and education for compliance (Bailey et al. 2015). Storage and calibration of supply vs. demand are very important for supplementation. Supplementation programme addresses few micronutrients only and does not address the poor-quality diet. Food fortification is another option of reducing MND. Fortification of salt with iodine, sugar and cooking oil with vitamin A and flour, dairy food, condiments, sugar and salt with iron is common practice (Bouis et al. 2017). In China, selenium-fortified salt and tea is being used against Keshan disease (Combs 2000). Folic acid-fortified wheat and maize is used to avoid neural tube defect caused due to folate deficiency (Centeno Tablante et al. 2019).

1.1 Advantages of Biofortification

During the last 60 years, agricultural research in developing countries has focussed on increasing the production and improving the availability of calorically dense staple crops. Similar efforts were not made for increasing the availability of micronutrient rich pulses, vegetables and consequently their prices have increased and these are not affordable to resource poor. Increased availability of biofortified crops and dietary diversification are vital for addressing the MND. Complementation of biofortification with supplementation and industrial fortification can alleviate MND. Biofortification is cost-effective and even resource poor can avail the benefits at marginal cost.

Biofortified varieties once developed can be evaluated for adaptation in new environments and geographies. Biofortification needs to be mainstreamed as a core breeding objective by international and national crop development programmes. Biofortified crops can be utilized by resource poor having no access to the diversified diet. The target micronutrient levels can meet the nutritional requirements of children and women. According to Hoddinott et al. (2013) benefit of US $17 may be gained for every dollar invested in biofortification. Cost-effectiveness of any intervention is based on crop, micronutrient and country. Supplements and industrialized fortified food provide a higher level of vitamins and minerals and biofortified crops ensure daily adequacy of micronutrients throughout the life.

2 Conventional Breeding

Crop biofortification is a multidisciplinary approach involving plant breeders, nutritionists and food technologists. Using conventional breeding the nutrient levels of staple crops can be increased to the target level without altering the agronomic traits and compromising yield levels. The steps in the development of micronutrient

dense varieties include the screening of primary and secondary genepool, pre-breeding and breeding for development of micronutrient dense lines and their testing at multilocation to assess G × E interactions (influence of environment on micronutrient expression). Breeding targets for each micronutrient is based on the consumption pattern of target population, nutrient bioavailability and losses during processing and storage.

Exploration of available genetic diversity (primary and secondary gene pool) for micronutrient concentration is the first step in crop improvements. Along with micronutrient concentration screening is also carried out for agronomic traits (yield, seed size, maturity duration, disease and insect resistance, etc.). The suitable genotypes identified are utilized in the hybridization programme for the development of mapping population for genetic and molecular studies and breeding material. Molecular markers have been identified in different crops linked to grain Fe, Zn and Se concentration using biparental or association mapping approach. Mapping populations, and markers identified for grain Fe, Zn and Se concentration in different crops are presented in Table 1.1. Molecular markers linked to micronutrients can facilitate in marker-assisted selection. The breeding material can be advanced rapidly by using offseason nursery and speed breeding. The existing varieties, pre varieties and finished germplasm products can be fast tracked to ensure their early delivery. Pre breeding is necessary when unadapted sources are used as donor in breeding programmes. Product enhancement activities and pre breeding are simultaneously carried out by most breeders. The micronutrient concentration, yield and resistance to biotic and abiotic stresses of the developed products can be assessed in multilocation trials (study of G × E interactions). The promising varieties identified are then tested in multiseason and multilocation trials for agronomic performance by national government agencies for release and notification.

2.1 Genetic Variation for Micronutrient Concentration

The genetic variation for micronutrient concentration has been studied in different crops. The most important sources include landraces, primitive cultivars and wild relatives. Fe range of 4–30 mg/kg and Zn range of 8–95 mg/kg, seed was reported by Yang et al. (1998) in rice. Gregorio et al. (2000) studied the core collection of brown rice and report Fe and Zn ranges of 6–24 and 14–58 mg/kg, respectively. Banerjee et al. (2010) reported Fe range of 4.8–22.7 mg/kg and Zn range of 13.95–41.73 mg/kg from the study of 46 rice accessions. Anuradha et al. (2012) evaluated 122 brown rice accessions and reported 6.2–71.6 mg/kg as a range for Fe. Jahan et al. (2013) reported a very high range (1.32–100.45 mg/kg) for Fe.

Wheat has been extensively studied for grain Fe and Zn concentration. Fe concentration range of 25–73 mg/kg and Zn concentration range of 25–92 mg/kg have been reported by Monasterio and Graham (2000). Clarke et al. (2002) reported similar range in durum wheat. Graham et al. (1999), Cakmak et al. (2000) and Bálint et al. (2001) studied wild relatives of wheat and reported higher Fe and Zn concentration in comparison to cultivated wheat. Hentschel et al. (2002) reported total

Table 1.1 Mapping populations, and markers identified for grain Fe, Zn and Se concentration in different crops

Crop	Population/lines/ cultivars/accession	No. of markers identified			Reference
		Identified for grain Fe conc.	Identified for grain Zn conc.	Identified for grain Se conc.	
Wheat	DH/Hanxuan 10 × Lumai 14		4 QTLs		Shi et al. (2008)
	DH/RAC875-2 × Cascades		4 QTLs		Genc et al. (2009)
	RIL/Langdon × G18-6	11 QTLs	6 QTLs		Peleg et al. (2009)
	RIL/Tb5088 × Tm14087	3 QTLs	2 QTLs		Tiwari et al. (2009)
	RIL/Xiaoyan 54 × Jing 411	2 QTLs	2 QTLs		Xu et al. (2012)
	RIL/Tabassi × Taifun	6 QTLs	2 QTLs		Roshanzamir et al. (2013)
	DH/Hanxuan × Lumai 14	4 QTLs			Shi et al. (2013)
	RIL/PBW343 × Kenya Swara		3 QTLs		Hao et al. (2014)
	RIL/SHW-L1 × Chuanmai 32	4 QTLs	4 QTLs	4 QTLs	Pu et al. (2014)
	RIL/P 1348449 × HUW 234	5 QTLs	5 QTLs		Srinivasa et al. (2014)
	DH/Berkut × Krichauff	1 QTL	2 QTLs		Tiwari et al. (2016)
	RIL/SeriM82 × SHW CWI76364	10 QTLs	3 QTLS		Crespo-Herrera et al. (2016)
	RIL/Louries × Bateleur	9 QTLs	12 QTLs		Crespo-Herrera et al. (2017)
	RIL/Bubo × Turtur	3 QTLs	4 QTLs		Crespo-Herrera et al. (2017)
	F2/WTSD91 × WN-64	3 QTLs	3 QTLs		Hussain et al. (2017)
	RIL/WH542 × PI94624	1 QTL	1 QTL		Krishnappa et al. (2017)
	RIL/Adana99 × 70711	8 QTLs	10 QTLs		Velu et al. (2017)
	RIL/TN18 × LM 6			7 QTLS	Wang et al. (2017)
	AM panel	3 QTLs	3 QTLs		Gorafi et al. (2018)

(continued)

Table 1.1 (continued)

Crop	Population/lines/ cultivars/accession	No. of markers identified			Reference
		Identified for grain Fe conc.	Identified for grain Zn conc.	Identified for grain Se conc.	
	RIL/SHW-L1 × Chuanmai 32			24 QTLs	Pu et al. (2018)
	AM panel		2 QTLs		Velu et al. (2018)
	RIL/Langdon × G 18-16			15 QTLs	Yan et al. (2018)
Rice	DH/IR64 × Azucena	3 QTLs	3 QTLs		Stangoulis et al. (2007)
	RIL/Zhenshan 97 × Minghui 63	2 QTLs	3 QTLs		Lu et al. (2008)
	BIL/Teqing × *O. rufipogon*	1 QTLs	3 QTLs		Garcia-Oliveira et al. (2009)
	Sasanishiki × Habataki	–	1 QTL		Ishikawa et al. (2010)
	RIL/Bala × Azucena	4 QTLS	1 QTL		Norton et al. (2010)
	DH/Zy08 × JX17		2 QTLs		Zhang et al. (2011)
	RIL/Madhukar × Swarna	2 QTLs	4 QTLs		Anuradha et al. (2012)
	F2/PAU × Palman 579	8 QTLs	3 QTLs		Kumar et al. (2014)
	AM panel	13 SSRs	2 SSRs		Nawaz et al. (2015)
	RIL/Swarna × Moroberekan	1 QTL			Indurkar et al. (2015)
	BILs/Ce258 × IR 75862 ZGX1 × IR 75862	1 QTL	4 QTLs		Xu et al. (2015)
	BILs/*O. sativa* × *O. rufipogon*	3 QTLs	6 QTLs		Hu et al. (2016)
	BRILs/Nipponbare × W 1627		4 QTLs		Ishikawa et al. (2017)
	BC2F2/Swarna × *O. nivara*	5 QTLs	3 QTLs		Swamy et al. (2018)
	DH/PSBRc 82 × Joryeongbyes PSBc × IR 69428	8 QTLs	1 QTL		Swami et al. (2018)
	DU/IR 64 × IR 69428 BR29 × IR 75862		8 QTLs		Descalsota-Empleo et al. (2019)

(continued)

Table 1.1 (continued)

Crop	Population/lines/ cultivars/accession	No. of markers identified			Reference
		Identified for grain Fe conc.	Identified for grain Zn conc.	Identified for grain Se conc.	
	BC2F5/RP-Bio226 × Sampada	2 QTLs	3 QTLS		Dixit et al. (2019)
	F4/PAU 201 × Palman	5 QTLs	1 QTL		Kumar et al. (2019)
	AM panel	7 QTLs	5 QTLs		Bollinedi et al. (2020)
	DH/IR05F102 × IR69428	5 QTLs	5 QTLS		Calayugan et al. (2020)
	DH/Hwaseonchal × Goami 2	1 QTL	1 QTL		Jeong et al. (2020)
	AM panel	2 QTLs	3 QTLs		Pradhan et al. (2020)
Pearlmillet	RIL/ICMS 8511-S1-17-2-1-1-B-P03 × AIMP 92901-S1-183-2-2-B-08	11 QTLs	8QTLs		Kumar et al. (2018)
	RIL/ICMB 841-P3 × 863B-P2	2 QTLs	2 QTLs		Kumar et al. (2016)
Maize	F 2:3/178 × P53	1 QTL	4 QTLs		Jin et al. (2013)
	RIL/B84 × Os6-2	3 QTLs	3 QTLs		Šimić et al. (2012)
Sorghum	RIL/296B × PVK 801	3 QTLs	3 QTLs		Kotla et al. (2019)
Chickpea	AM panel	4 SNP	5 SNPs		Diapari et al. (2014)
	RIL/ICC 4958 × ICC 8261	8 QTLs	8 QTLs		Upadhyaya et al. (2016)
Lentil	AM panel	2 SNPs	1 SNP		Khazaei et al. (2017)
	AM panel	4 SSRs	3 SSRs		Singh et al. (2017)
	AM panel	2 SSRs	3 SSRs		Kumar et al. (2018)
Common beans	RIL/AND 696 × GI 9833	1 QTL	1 QTL		Cichy et al. (2009)
	RIL/DOR 363 × GI 9833	13 QTLs	13 QTLs		Blair et al. (2010)
	AM panel	6 SNPs	6 SNPs		Katuuramu et al. (2018)
	7 populations	12 meta QTLs			Izquierdo et al. (2018)

(continued)

Table 1.1 (continued)

Crop	Population/lines/ cultivars/accession	No. of markers identified			Reference
		Identified for grain Fe conc.	Identified for grain Zn conc.	Identified for grain Se conc.	
Pea	AM panel	1 EST SSR	–		Kwon et al. (2012)
	AM panel	9 SNPs	2 SNPs		Diapari et al. (2015)
Soybean	F2:4 lines/Anoka × A7	2 genes	–		Peiffer et al. (2012)
	–	1 QTLs	–		King et al. (2013)

carotenoids content of 200 μg/100 g for four wheat varieties. Cakmak et al. (2004) reported Fe range of 14–190 mg/kg and Zn range of 15–109 mg/kg. They reported that wild species are an important source for increasing Fe and Zn concentration in wheat grains. Hidalgo et al. (2006) reported carotenoids content of 54 cultivars of Einkorn wheat, 6 durum wheat varieties and 5 bread wheat cultivars and reported carotenoids concentration of 320 μg/100 g, 195 μg/100 g and 841 μg/100 g, respectively. Morgounov et al. (2007) studied 60 germplasm lines and reported Fe range of 25–56 mg/kg and Zn range of 20–39 mg/kg. Velu et al. (2011) recorded 25–56 mg/kg Fe and 26–65 mg/kg Zn in wheat accessions. They reported that the genotypes with high level of micronutrients were unadapted with low yield level. Badakhshan et al. (2013) studied 81 cultivars of bread wheat and reported Fe range of 41.4–67.7 mg/kg and Zn range of 36.4–73.8 mg/kg. Goel et al. (2018) studied wheat landraces of India and reported modest Fe range of 32.7–54.5 mg/kg and Zn range of 15.8–66 mg/kg. Khokhar et al. (2020) reported Zn range of 24–49 mg/kg from the study of 245 landraces.

Pearl millet is a rich source of micronutrients among the cereals. The variation for grain Fe (31–61 mg/kg) and Zn (32–54 mg/kg) in pearl millet has been reported by Velu et al. (2007), Gupta et al. (2009), Govindaraj et al. (2013) and Rai et al. (2013). Pucher et al. (2014) evaluated 72 pearl millet accessions from West and Central Africa in Niger and reported Fe range of 24.2–48.8 and Zn range of 19.8–43.4 mg/kg. Evaluation of 225 pearl millet accessions in Sudan (Bashir et al. 2014) revealed Fe range of 19.7–86.4 mg/kg and Zn range of 13.5–82.4 mg/kg. Carotenoids in the range of 0.5–3.4 μg/g for maize hybrids has been reported by Egesel et al. (2003) and range of 0.7–4.7 μg/g for kernel β-carotene has been reported by Menkir et al. (2008). Kernel β-carotene range of 0.01–1.72 μg/g for a set of Chinese maize inbreds has been reported by Chander et al. (2008). Prasanna et al. (2011) reported kernel Fe range of 11.28–60.4 mg/kg and Zn range of 15.14–52.95 in maize kernel. Queiroz et al. (2011) reported Fe range of 12.2–36.7 mg/kg and Zn range of 17.5–42 mg/kg by evaluating 22 diverse tropical inbreds. Vignesh et al. (2012) reported range of 0.02–16.50 μg/g for kernel β-carotene for 105 diverse maize inbreds.

Ma et al. (2004) estimated Fe range of 21–83 mg/kg in the barley core collection. In sorghum, Reddy et al. (2005) reported Fe in the range of 20–37 mg/kg and Zn in the range of 13–31 mg/kg. Islam et al. (2002) reported Fe range of 35–92 mg/kg and Zn range of 21–59 mg/kg in beans. Fe range of 48–74 mg/kg and Zn range of 17–28 mg/kg in beans were reported by Ariza-Nieto et al. (2007). A wide range of variability for grain Fe and Zn concentration in lentil has been estimated by Singh et al. (2017). Fe concentration in lentil seed varied from 34.4–119.5 mg/kg seed and Zn from 12.3–78.75 mg/kg seed.

3 Transgenic Approach

In crops with low diversity for the desired micronutrient in gene pool, transgenic approach is a viable option for producing biofortified varieties possessing desired concentration of micronutrient and agronomic traits. Rice has been improved for vitamin A using daffodil *Phytoene synthase* and *Erwinia uredovora phytoene desaturase* (Ye et al. 2000), maize *phytoene synthase* (Paine et al. 2005) and *daffodil phytoene synthase* and *lycopene β-cyclase* (Beyer et al. 2002). Fe biofortification in rice has been reported using overexpression of soybean *ferritin gene Soyfer H-1* (Goto et al. 1999), *phaseolus ferritin* (Lucca et al. 2001), *ferritin* (Masuda et al. 2012, 2013) and *OsNAS2* (Johnson et al. 2011). Zn enhancement in rice has been carried out using barley *HvNAS1* gene (Masuda et al. 2009), soybean *ferritin, Aspergillus flavus phytase, OsNAS1* (Wirth et al. 2009) and *OsNAS2* (Johnson et al. 2011). Vitamin A biofortification in wheat has been reported by Cong et al. (2009) (maize *psy1* gene encoding *phytoene synthase*, *bacterial crtI*) and Wang et al. (2014) (*Crt*B *or Crt*I).

Wheat biofortification for Fe was reported by Drakakaki et al. (2000) (soybean *ferritin*) and Borg et al. (2012) (overexpression of *TaFer1-A*). In maize vitamin A-rich transgenics have been produced using bacterial *crtB and crtI* (Aluru et al. 2008) and maize *psy1* (Naqvi et al. 2009). In cassava, Welsch et al. (2010) reported the development of vitamin A-rich transgenic using bacterial *crtB.* Ravanello et al. (2003) developed vitamin A-rich canola using *crtB and crtI*. Transgenic varieties possess great potential but their release for cultivation depends on approval for national biosafety and regulatory processes. The release of golden rice is delayed due to highly risk averse regulatory approval processes (Wesseler and Zilberman 2014). Golden rice has been approved as safe for human consumption by regulators in the Philippines and Bangladesh.

4 Efficacy of Biofortified Crops

The reported biofortified varieties released in different crops through conventional breeding are presented in Table 1.2. The evidence for efficacy of biofortified crops is generated by nutritionists. Biofortified crops are processed, packed, stored and cooked before consumption. Detainment of micronutrient during these processes is

Table 1.2 Status of release of biofortified crop varieties

Micronutrient	Crop	Variety/hybrid	Status	Country	Reference
Iron	Rice	IR68144-3B-2-2-3	Improved line	India	Garg et al. (2018)
		Jalmagna	Traditional variety	India	Gregorio et al. (2000)
	Pearl millet	Dhanshakti ICMH 1201	Released	India	Rai et al. (2013)
		Chakli Hybrids: ICMH 1202, ICMH 1203, ICMH 1301	Released	India	Govindaraj et al. (2019)
	Sorghum	ICSR14001, ICSH 14002 Hybrids: ICSA 661 × ICSR 196 ICSA 318 × ICSR 94 ICSA 336 × IS 3760	Released	India	Garg et al. (2018)
		Parbhani Shakti	Released	India	https://www.icrisat.org/india-gets-its-first-biofortified-sorghum/
		12KNICSV (Deko)-188, 12KNICSV-22 (Zabuwa)	Released	Nigeria	Garg et al. (2018)
	Lentil	IPL 220 L 4717	Released	India	Yadava et al. (2018)
	Cowpea	Pant Lobia 1, Pant Lobia 2, Pant Lobia 3, Pant Lobia 4	Released	GBPUA&T	Yadava et al. (2018)
	Bean	ICTA Superchiva, ICTA Peten, ICTA Chorti	Released	Guatemala	Andersson et al. (2017)
		CorpoicaRojo 39, CorpoicaRojo 43, Bio 101, Bio 107	Released	Columbia	Andersson et al. (2017)

(continued)

Table 1.2 (continued)

Micronutrient	Crop	Variety/hybrid	Status	Country	Reference
		MOORE 88002, RWR 2154, RWR 2245, MAC 44 and Nyiramuhondo	Released	Uganda	https://www.icrisat.org/release-of-biofortified-bean-varieties-in-uganda/
		COD MLV 059, PVA 1438, Nain de Kyondo, COD MLB 032, Cuarentino, COD MLB 001, HM 21-7, RWR 2245, VCB 81013	Released	Democratic Republic of Congo	Andersson et al. (2017)
		BRS Agreste, BRS Pontal, BRS 9435 Cometa	Released	Brazil	Andersson et al. (2017)
		Fortaleza	Released	Bolivia	Andersson et al. (2017)
		MIB (NUT) 396-33, MIB (NUT) 397-72	Released	Honduras	Andersson et al. (2017)
		INTA Ferroso, INTA Nutritivo	Released	Nicaragua	Andersson et al. (2017)
		IDIAP NUA 24, IDIAP NUA 27	Released	Panama	Andersson et al. (2017)
		MAC 44, RWV 1129, CAB 2, RWR 2245, RWR 2154, RWV 3316, RWV 3006, RWV 3317, MAC 42, RWV 2887	Released	Rwanda	Andersson et al. (2017)
Iron and zinc	Wheat	WB2	Released	India	Yadava et al. (2018)
	Maize	HHB 229 AHB 1200	Released	India	Yadava et al. (2018)
	Pomegranate	Solapur Lal	Released	India	Yadava et al. (2018)

	Rice	BRRIdhan62, BRRIdhan64, BBRIdhan72	Released	Bangladesh	HarvestPlus progress report (2014) https://www.harvestplus.org/sites/default/files/publications/Biofortification_Progress_Briefs_August2014_WEB_2_0.pdf
	Lentil	Barimasur-4, Barimasur-5, Barimasur-6, Barimasur-7, Barimasur-8	Released	ICARDA	HarvestPlus progress report (2014) https://www.harvestplus.org/sites/default/files/publications/Biofortification_Progress_Briefs_August2014_WEB_2_0.pdf
		ILL 7723, Khajurah-1, Shital, Sisir, Sekhar, Simal	Released	Nepal	Andersson et al. (2017)
		Alemaya	Released	Ethiopia	Andersson et al. (2017)
		Idlib-2, Idlib-3	Released	Syria	Andersson et al. (2017)
	Beans	RWR 2245, RWR 2154, MAC 42, MAC 44, CAB 2, RWV 1129, RWV 3006, RWV 3316, RWV 3317, RWV 2887	Released	Rwanda	Andersson et al. (2017)
Zinc	Wheat	BHU 1, BHU 2, BHU 3, BHU 5, BHU 6, BHU 17, BHU 18	Released		Yadava et al. (2018)
		PBW1Zn	Released	India	Yadava et al. (2018)
		NR 419, 42, 421, Zincol	Released	CIMMYT CIAT	HarvestPlus progress report (2014) Biofortification_Progress_Briefs_August2014_WEB_2_0.pdf
	Rice	Jalmagna	Released	India	Gregorio et al. (2000)
	Maize	ICTA HB-18, ICTA B-15	Released	Mexico	Maqbool and Beshir (2019)
	Maize	BIO-MZN01	Released	Colombia	Maqbool and Beshir (2019)
	Bean	BIO-101, BIO 107	Released	Colombia	Beintema et al. (2018)
Protein	Rice		Released	NRRI	

(continued)

Table 1.2 (continued)

Micronutrient	Crop	Variety/hybrid	Status	Country	Reference
		CR Dhan 310, CR Dhan 311			http://pib.nic.in/PressReleaseIframePage.aspx?PRID=1566398
Protein and zinc	Wheat	PusaTejas (HI 8759)	Released	India	Yadava et al. (2018)
Protein, iron, zinc		Pusa Ujala (HI 1605), MACS 4028 (d)	Released	India	Yadava et al. (2018)
Low erucic acid	Mustard	Pusa Mustard 30	Released	India	Yadava et al. (2018)
Low erucic acid and glucosinolate		Pusa Double Zero Mustard 31	Released	India	Yadava et al. (2018)
β Carotene	Wheat	HI 8627	Released	India	Garg et al. (2018)
	Sweet poato	Bhu Sona	Release	India	Yadava et al. (2018)
	Cauliflower	Pusa Beta Kesari 1	Release	India	Yadava et al. (2018)
Anthocyanin	Wheat	NABIMG-9, NABIMG-10, NABIMG-11	Germplasm registered	NABI	Garg et al. (2018)
		Indigo	Released	Austria	Havrlentova et al. (2014)
		PS Karkulka	Released	Slovakia	Havrlentova et al. (2014)
		Black grained wheat	Released	China	Havrlentova et al. (2014)
	Sweet potato	Bhu Krishna	Release	India	Yadava et al. (2018)
QPM (lysine and tryptophan)	Maize	CML 176, CML 176 × CML 186, HQPM-1, HQPM-4, HQPM-5,	Released	Mexico	Garg et al. (2018)

		HQPM-7, VivekQPM-9, FQH-4567			
		Pusa HM4 improved, Pusa HM8 improved, Pusa HM9 improved	Released	India	Yadava et al. (2018)
		Pusa HM4, Pusa HM 8, Pusa HM 9	Released	India	Hossain et al. (2018)
		CML 140, CML 194, P70	Released	China	Garg et al. (2018)
		CML 161 × CML 165	Released	Vietnam	Garg et al. (2018)
		CML 142 × CML 176, CML 142 × CML 150, CML 176 × CML 170, CML 186 × CML 149, CML 176 × CML 186	Released	Mexico	Garg et al. (2018)
		QS-7705	Released	South Africa	Garg et al. (2018)
		GH 132-28	Released	Ghana	Garg et al. (2018)
		Obatampa	Released	Guinea	Garg et al. (2018)
		Obatampa	Released	Benin	Garg et al. (2018)
		Obangaina	Released	Uganda	Garg et al. (2018)
		Susma	Released	Mozambique	Garg et al. (2018)
		BR-451, BR-473	Released	Brazil	Garg et al. (2018)
		FONIAP	Released	Venezuela	Garg et al. (2018)
		INIA	Released	Peru	Garg et al. (2018)
		ICA	Released	Colombia	Garg et al. (2018)
		HQ-31	Released	Honduras	Garg et al. (2018)
		HQ-61	Released	El Salvador	Garg et al. (2018)
		HB-Proticta	Released	Guatemala	Garg et al. (2018)

(continued)

Table 1.2 (continued)

Micronutrient	Crop	Variety/hybrid	Status	Country	Reference
		NB-Nutrinta, HQ INTA-993	Released	Nicaragua	Garg et al. (2018)
High provitamin-A, high tryptophan and lysine		Pusa Vivek QPM improved	Released	India	Yadava et al. (2018)
		Shakti-1 (composite) and hybrids, namely Shaktiman-1, Shaktiman-2, HQPM-1, Shaktiman-3, Shaktiman-4, HQPM-5, HQPM-7, Vivek QPM-9, HQPM-4, Pratap QPM Hybrid-1 and Shaktiman-5	Released	India	Yadava et al. (2018)
QPM plus Provitamin A	Maize	Pusa Vivek QPM 9	Released	India	Yadava et al. (2018)
Kunitz trypsin inhibitor	Soybean	NRC 127	Released	India	Yadava et al. (2018)
Vitamin A	Sorghum	UMUCASS 36, UMUCASS 37, UMUCASS 38	Released	Nigeria	https://www.iita.org/news-item/nigeria-releases-cassava-higher-pro-vitamin-fight-micronutrientdeficiency/
	Maize	A0905-28 and A0905-32	Released	IITA	Onuegbu et al. (2017)
	Sweet potato	Ejumula, Kakamega, Vita, Kabode, NASPOT 7, NASPOT 8, NASPOT 9 O, NASPOT 10 O, NASPOT 12 O, NASPOT 13 O and Dimbuka-Bukululа	Released	Uganda	Mwanga et al. (2007, 2009, 2016)
	Cassava		Released	Nigeria	Andersson et al. (2017)

		TMS01/1368-UMUCASS36, TMS01/1412-UMUCASS 37, TMS01/1371-UMUCASS 37, UMUCASS 38, NR 07/0220-UMUCASS 44, TMS07/0593-UMUCASS 45 and TMS07/539-UMUCASS 46			
		Kindisa (TMS2001/1661)	Released	Democratic Republic of Congo	Andersson et al. (2017)
	Sweet potato	Olympia, Twatasha, Kokota, Chiwoko, Zambezi	Released	Zambia	Andersson et al. (2017)
ProvitaminA	Maize	Ife Hyb 3, Ife Hyb 4, Sammaz 38, Sammaz 39, Sammaz 43, Sammaz 44,	Released	Nigeria	Andersson et al. (2017)
		GV671A, GV672A, GV673A, GV665A, GV662A, GV664A, GV671A, GV672A, GV673A, GV665A, GV662A, GV664A	Released	Zambia	HarvestPlus progress report (2014) https://www.harvestplus.org/sites/default/files/publications/Biofortification_Progress_Briefs_August2014_WEB_2_0.pdf
		Sam Vita 4-A, Sam Vita 4-B, Muibaki 3, Muibaki 2, Muibaki GV662	Released	Democratic Republic of Congo	Andersson et al. (2017)

(continued)

Table 1.2 (continued)

Micronutrient	Crop	Variety/hybrid	Status	Country	Reference
		ZS242(HP1005), ZS244 (HP1301), ZS246 (HP1302), ZS248(HP1303)	Released	Zimbawe	Andersson et al. (2017)
		MH39A, MH40A, MH42A, MH43A	Released	Malawi	Andersson et al. (2017)
		Nafama, Abebe, Duba, Kodialan, Dakan	Released	Mail	Andersson et al. (2017)
		BRS 4104	Released	Brazil	Released
	Cowpea	NR07/0220, TMS07/0593, TMS07/0539, TMS01/1412, TMS01/1368, TMS 1/1371	Released	Nigeria	Andersson et al. (2017)
	Banana and plantation	Apantu, Bira, Pelipita, Toó, Lahi	Released	Burundi	Andersson et al. (2017)
		Apantu, Bira, Pelipita, Toó, Lahi	Released	Democratic Republic of Congo	Andersson et al. (2017)

assessed. The absorption of micronutrient is vital for improvement of nutritional status. The measurement of micronutrient absorption by human body is the next step. Randomized controlled efficacy trial is conducted and the study of functional indicators of micronutrient status is carried out to assess the impact of intake of biofortified crop.

4.1 Iron-Rich Crops

The nutritional efficacy trials have been conducted in beans and pearl millets. These crops have been biofortified for grain Fe.

Beans: Beans are an important part of diet in Central and South America and Africa. Fe breeding target in beans is 94 ppm. The bioavailability is reported to be in the range of 3.8–7.3% when beans are consumed with rice or potatoes (Petry et al. 2012, 2014, 2016). Fe absorbed from beans is in the range of 234–431 μg which is 30% of requirement of normal women (FAO/WHO 2004). Increasing Fe without an increase in phytic acid (PA) will improve the bioavailability. However, low PA beans are reported to be possessing hemagglutinin residues and poor cooking quality (Petry et al. 2016). Phytic acid (PA) concentration increases with increase in Fe concentration in beans and high PA limits Fe bioavailability (5). Haas et al. (2017) reported increase in haemoglobin and total body iron of iron exhausted university women in Rwanda consuming biofortified beans for 4.5 months.

Pearl millet: Pearl millet is the staple food in India, West and Central Africa (Rao et al. 2006). Fe breeding target is 77 ppm. Iron biofortified pearl millet possesses almost thrice Fe concentration. Beninese pearl millet paste (60 g pearl millet) of control and biofortified pearl millet was fed to 20 Beneinese women with vegetable/okra sauce for 5 days. The mean iron absorption of 7.5% was recorded and the amount of Fe absorbed from biofortified pearl millet was twice than control/non-biofortified pearl millet (Cercamondi et al. 2013). The results revealed that women of reproductive age in North Benin can meet 70% of their Fe requirement by consuming 180 g of biofortified pearl millet every day. The study by Kodkany et al. (2013) in which three different test meals were fed to school children revealed that iron biofortified pearl millet can ensure adequate Fe required for children (the Fe bioavailability was 6–9%). The consumption of pearl millet flat bread twice a day improved serum ferritin and total body iron of iron-deficient students of secondary school in Maharashtra, India (Finkelstein et al. 2015).

Rice: Nine-month randomized feeding trial was conducted on 192 religious sisters in Philippines. Participants consumed biofortified rice (3.2 mg/kg) or local variety (0.57 mg/kg). The consumption of biofortified rice increased serum ferritin (Haas et al. 2005).

4.2 Zinc-Rich Crops

Rice: Rice is an important source of energy in Asia. The breeding target for Zn in rice is 28 ppm. Zn is evenly distributed in rice grains. Juliano (1985) reported loss up to 10% in Zn of milled rice due to washing before cooking. Removal of excess water from boiled rice removes 10–14% Zn (Dipti 2012). Bioavailability and bioefficacy evidence is yet to be reported. Serum Zn concentration is not sensitive to low additional Zn intake. Sensitive biochemical indicators are required to judge the impact of Zn intervention on human health.

Wheat: Wheat is the most important staple food. The breeding target for Zn in wheat is 37 ppm. The duration and intensity of milling is proportional to milling losses. During milling, phytates are reduced increasing the bioavailability. Rosado et al. (2009) reported that Zn absorption from biofortified wheat is greater than from non-biofortified wheat.

4.3 Vitamin A-Rich Crops

Orange sweet potato, cassava and maize are rich in β-carotene and this gives the yellow colour to biofortified crops. Human body converts β-carotene into vitamin A. Conversion of provitamin A to retinol determines the bioavailability of vitamin A. Studies have revealed that the consumption of vitamin A biofortified crops improves serum retinol.

Orange sweet potato (OSP): The target for provitamin A biofortification in orange sweet potato is 30 ppm. Significant increase in vitamin A has been reported due to consumption of OSP (Haskell et al. 2004; Van Jaarsveld et al. 2005; Low et al. 2007). The evidence for the effectiveness of biofortified OSP in improving vitamin A status was gathered from randomized controlled trial conducted in Uganda and Mozambique. As a result of introduction and intake of OSP in Uganda the vitamin A indicated reduction on low serum retinol by 9% (Hotz et al. 2012). Biofortified OSP in Mozambique improved child health and minimized duration and frequency of diarrhoea in children below 5 years (Jones and De Brauw 2015).

Cassava: The target for provitamin A biofortification in cassava is 15 ppm. The efficacy trial of provitamin A cassava was conducted in Kenya on children aged between 5 and 13 years. Serum retinol and β carotene revealed improvement in vitamin A status (Talsma et al. 2016).

Maize: The target for provitamin A biofortification in maize is 15 ppm. Gannon et al. (2014) reported an increase in total body store of vitamin A in 5–7-year-old children as a result of consumption of provitamin A biofortified maize for 3 months. Consumption of orange maize improved vision of vitamin A-deficient children (Palmer et al. 2016).

5 National Programmes Working on Crop Biofortification

Several countries have strong national programmes for biofortification of a range of food crops.

5.1 India

Department of Biotechnology and the Indian Council of Agricultural Research have joined hand for development of biofortified varieties of wheat, rice and maize. HarvestPlus is collaborating with ICRISAT for sorghum and pearl millet, Indian Agricultural Research Institute and Indian Institute of Pulses Research for lentil and Directorate of Rice Research for rice.

5.2 Brazil

BIOFORT programme operated by Brazilian Agricultural Research Corporation (EMBRAPA) focused on biofortification of maize, wheat, rice, sweet potato, Cassava, cowpea, bean and pumpkin. Significant progress has been made in cowpea, cassava and maize, and a number of biofortified varieties have been released.

5.3 China

Biofortification programme targeting provitamin A, iron and zinc in sweet potato, wheat, rice and maize was initiated 15 years back. Biofortified wheat, rice and sweet potato varieties have been released for commercial cultivation.

6 Future Strategies

Considering the impact of MND it is essential to integrate biofortification as a key objective in breeding programmes. The role of micronutrients for sustaining and improving human health and ensuring suitable economic returns on investments in biofortification must be recognized by the officials managing the International and national crop improvement programmes. Appropriate budgetary provisions are required for strengthening the biofortification programme across the crops.

Agricultural institutions must identify the minimum level of minerals and vitamins for the release of new varieties of different crops. Indian Council of Agricultural Research, New Delhi has taken lead and fixed micronutrient level as 42 ppm for Fe and 32 ppm for Zn for promotion and release of all pearl millet varieties, hybrids, synthetics and composites in India. Standards are required for other crops. In the recent years, molecular markers have been developed for rapid introgression of QTLs controlling micronutrient concentration. Building consumer

demand is a key challenge. Rural and urban consumers must recognize the value of micronutrients and ensure adequate demand for biofortified crops. Governments can ensure the availability of biofortified crops at subsidized rates for micronutrient-deficient rural poor.

7 Conclusion

HarvestPlus is leading the global biofortification programme. The focus has shifted to delivery after development of varieties in important staple crops. Scaling of cultivation of biofortified crops will require expansion of existing partnership and building new partnership. HarvestPlus in collaboration with national programmes is training extension staff on nutrition message and technical package for delivery programme. Strong financial support (from international and national funding agencies) is required for delivery of biofortified crops to those who need it most. Public and private sector have to join hands for mainstreaming biofortification. Food processors and retailers need to add biofortified crops in their products.

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Historical Overview of Biofortification in Crop Plants and Its Implications

2

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Abstract

Development of a crop variety having a significantly higher concentration of a certain nutrient than the normal crop which is being grown is known as biofortification. Staples are the world's primary source of dietary micronutrients particularly for the rural population living in developing economies. Fortification of food product has very ancient history which dates back to 1920s while biofortification of staple crops is relatively novel strategy which is being practiced across different parts of the world since 1990s. During the last three decades, good progress has been made in the nutritional enrichment or biofortification of various staple crops like sweet potato and maize (provitamin A), beans and lentils (iron), rice (zinc), etc., using both conventional and molecular tools. The biofortified crops are now a reality which are being grown in 35 countries and consumed by more than 40 million low-income population in developing countries who cannot afford a diverse diet. This has resulted in reducing the magnitude of hidden hunger across the globe especially in the target population. Biofortification has an advantage that after initial developmental investment, no other costs are involved, making this strategy sustainable. Additionally, the Copenhagen Consensus has also considered biofortification as a sound investment and for every dollar invested, in terms of health and other productivity benefits. In this backdrop, this chapter focuses on two aspects: (1) Historical developments in the biofortification of various

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staple crops across the world; and (2) Key implications of the biofortification programme on the target population.

Keywords

Biofortification progress · Biofortification target · Biofortified varieties · Genesis · HarvestPlus · Hidden hunger

1 Introduction

A key challenge of contemporary world is that nearly one-sixth of the global population is suffering from hunger and among these, more than 2 billion are suffering from a different form of hunger called 'hidden hunger', especially in sub-Saharan Africa and South Asia (FAO et al. 2015). 'Hidden hunger' occurs when people consume foods lacking enough nutrients like essential vitamins and micronutrients such as iron, zinc and vitamin A, which are needed for healthy and productive lives (Bouis and Welch 2010). Micronutrients are the class of essential nutrients which are required by human body in very small quantity for the normal growth and development (Prashanth et al. 2015; White and Broadley 2005). The deficiencies of micronutrients are known to affect nearly 38% of pregnant women and 43% of pre-school children, globally. Childhood stunting and anaemia (30% of global population) are mainly caused by Zn and Fe deficiencies, respectively and are prevalent in many developing countries of Africa and South-East Asia (Branca and Ferrari 2002; Stevens et al. 2013; Brotanek et al. 2005).

For adults, the RDA (recommended daily allowance) based on US standard and daily reference nutrient intake (RNI) based on UK for zinc are 8.0–13.0 and 7.0–13.0 mg, respectively (Department of Health-UK 1991; Institute of Medicine-USA 2001). For vitamin A the RDA for adult men and women is 900 and 700 μg retinol activity equivalents (RAE)/day, respectively, while for Fe this is 8 mg/day for all age groups of men and postmenopausal women, while for premenopausal women this is 18 mg/day (DRI 2000). The diets of almost one-third of the people across globe lack required concentration of micronutrients in their food source (White and Broadley 2009; Bouis and Welch 2010; Stein 2010; Sayre et al. 2011) which add to the total global 'hidden hunger' disease burden (WHO 2002; Hotz and Brown 2004; White and Broadley 2011).

It was found that the first 1000 days of the child's development i.e., from the pregnancy of mother till child turns 2 years old was very crucial and child with hidden hunger expresses poor mental and physical development which may lead to blindness, various deficiency diseases or even death (Listman et al. 2019; CGIAR 2019). Overall, the symptoms of hidden hunger are quite pronounced in women and children under the age of five and is more widespread in developing countries as the population in these countries are relying more on staple crops like wheat, maize, lentils, beans, sweet potato, rice, etc., and are not having enough access to nutrient-rich foods and thus prone to suffer from vitamin A, iron and zinc deficiencies (Listman et al. 2019; CGIAR 2019).

The best approach to overcome the micronutrient deficiency is the consumption of micronutrient-rich balance diet and this can also be managed by supplementation, food fortification and biofortification (Kumar et al. 2019b). Fortification of product is being practiced since very long and table salt is the first product which is used for the iodine fortification during early 1920s in Switzerland and United States (Braverman et al. 2012). A long history of food fortification is known for butter, margarine and sugar (vitamin A), salt (iodine, fluoride), milk (vitamins), etc., across the world. Moreover, mandatory food fortification was found more effective in terms of its impact on the population over voluntary fortification. In India, the mandatory fortification begun in 1953 as vitamin A and D fortification of hydrogenated vegetable oil (Liu et al. 2014). Further, during 1998, table salt fortification was mandated with iodine to control Goitre and wheat flour fortification in West Bengal (2000) which was followed by Andaman and Nicobar Islands. Folic acid fortification of wheat flour has shown to reduce the neural tube defects or the defect in the brain and spine of the new-born babies by nearly 46% (Blencowe et al. 2010). It was estimated that till the year 2016, nearly 61 countries are using wheat flour fortified with folic acid (Lockyer et al. 2018). As per the 2017 estimates, 80% of wheat flour, 54% of rice and 29% of maize flour which was available for the human consumption are industrially milled and of these only 31%, 1% and 65%, respectively, are fortified with some essential minerals and/or vitamins (Food Fortification Initiative 2018).

In addition, the ongoing public health interventions as practiced by different governments like supplementation and industrial fortification are not found very successful as they require infrastructure, purchasing power, market access, etc., which is often not in the economic reach of the neediest people residing in the remote villages. Further, the vitamin A supplementation programs could cover only 58% of the population (UNICEF 2007), while 'Nutritional Anaemia Control Programme' which was started in the year 1970 in India was also not found very effective in terms of its impact (Vijayaraghavan 2002). Similarly, the iron-folate supplements could cover a meagre of nearly 30% of the total pregnant women and only 10% of the adolescent girls (Stein 2006; Mayer et al. 2008). Moreover, in developing countries, most of the poor and farming communities are chiefly dependent on their own farm produce and not on the industrially produce processed or fortified produce (Kumar et al. 2019b).

Thus, one possible solution considered was 'biofortification' or enhancing the vitamin and mineral content in the staple food crops through concerted research efforts as the resource-poor families across the globe are mainly dependent on such crops (Bouis et al. 2011, 2013; Gilligan 2012), thus making these as ideal crops for biofortification (Listman et al. 2019; CGIAR 2019). The prime target of biofortification in present scenario is Fe, Zn and vit. A, while other key targets include increased essential amino acids, more oleic acid, etc. (Hirschi 2009). They explored the ways to enrich the micronutrient content of the staple crops by the application of various fertilizers (agronomic interventions), through breeding approaches, genetic modification (Kumar et al. 2019a), or microbiological approaches (Cakmak 2008; Cakmak et al. 2004; Graham et al. 2007; Kumar et al. 2016; Pfeiffer and McClafferty 2007). Genetic enrichment has two key comparative

advantages in terms of its (1) long-term cost-effectiveness and (2) ability to reach the neediest rural populations easily (Bouis and Saltzman 2017).

Biofortification is different from the conventional fortification as it aims to improve the nutrient content of the crop when it is growing and not by manual means during processing. Biofortification is therefore considered as a way to reach the target populations where supplementation and/or conventional fortification is considered very difficult or very limited to implement (WHO 2019). Thus, introducing staple crops with increased nutrition may result in an immense influence, as it depends on improving an already existing food supply (Nestel et al. 2006). In this backdrop, this chapter covers the historical developments in biofortification and its implications in terms of product development and its reach to the target population at global level.

2 Genesis of Biofortification of Crops

2.1 Conceptualization of Biofortification (1950–1990)

Conceptually, biofortification has been around since the Green Revolution (1966–1985) (Pingali 2012). During 1950s and 1960s, Dr. Nevin Scrimshaw, a World Food Prize laureate, has demonstrated the impact of supplements like iron, iodine and vitamin A on the health of poor children in developing countries which prompted the CGIAR (Consultative Group on International Agricultural Research) to initiate the systematic work on biofortification (Croft-Cusworth 2018).

2.2 Realization of Biofortification Research (1990–2000)

'Hidden hunger' which is also known as 'micronutrient malnutrition' (MNM) came into the limelight during mid-1980s (Allen 2000). The World Summit for Children which was held during 1990 became the landmark event in the fight for hidden hunger through staple food crops. The three goals of this summit focused on the elimination or reduction of iron, zinc and vitamin A deficiencies by the year 2000 by targeting the traditional public health intervention strategies and was facilitated by the UN with support from UNICEF (United Nations International Children's Fund), the World Bank, WHO (World Health Organization), FAO (Food and Agriculture Organization), UNDP (United Nations Development Programme), CIDA (Canadian International Development Agency) and USAID (United States Agency for International Development) (Allen et al. 2006; UNICEF 2007; Darnton-Hill et al. 2005; Sanghvi et al. 2007; McLean et al. 2007).

Dr. Howarth Bouis, a World Food Prize laureate while working as an economist at the International Food Policy Research Institute (IFPRI) in Washington DC, researched on the food consumption pattern of economically weak households in Asia. He studied the nutrient intake pattern which gets severely influenced by the food prices and household income. His results showed that the differences in food

intake between the rich and the poor can be clearly understood by the quantity and the kind of non-staple food consumed by these two groups. It was observed that the extra vitamins and mineral contents which is present in these non-staple foods and animal products were highly correlated than just the calorie intake. This results in better health in terms of overall growth of a person including their height and less frequent illnesses. Thus, he could conclude that the mineral and vitamin deficiencies are the key constraints to better nutrition and, in turn to healthy and productive lives. It was estimated that through biofortification of the staple crops, the deprived population particularly the women and children can be supplemented with 30–100% of their daily requirement of various micro-nutrients, who often suffer the most from such deficiencies. Thus, he firmly proposed that the biofortification can be used as a tool to improve the health and productivity of the deprived population. He found the intersection between nutrition and agriculture by increasing the vitamin and mineral content of the staple food crops grown and consumed by smallholder farmers as biofortification which would ultimately help solving the global problem of hidden hunger (HarvestPlus 2016a).

With this information in hand, Dr. Bouis in the early 1990s started working on this aspect and collaborated with Robin Graham and Ross Welch, who received funds from DANIDA (Danish International Development Agency) and the Australian Center for International Agricultural Research for the identification of nutrient-rich genotypes as breeding parents (Graham et al. 2001). Afterwards, a number of funding agencies such as the CGIAR, the Asian Development Bank (ADB), the Bill and Melinda Gates Foundation, the World Bank, the US and UK governments and the European Union (EU) funded a number of research projects on biofortification of staple crops. Although the idea of biofortification of the staple crops initially faced a lot of suspicion, but in due course of time the research pertaining to biofortification especially in developing countries had succeeded in addressing the hidden hunger and energy requirements in the economically deprived population (Croft-Cusworth 2018).

2.3 Proof of Concept and Biofortified Product Delivery (2001–2020)

The term ‘biofortification’ was coined in the year 2001 by Steve Beebe, a bean researcher at CIAT (International Center for Tropical Agriculture), Colombia. Further, in 2001, the General Assembly of the UN adopted the Millennium Development Goals (MDGs) resolution, aiming to fight micronutrient malnutrition an integral component (UNGA 2000). The three of the eight MDGs which focused on the nutrition were (1) eradication of extreme poverty and hunger; (4) reduction of child mortality; and (5) improvement of maternal health (UNSCN 2004). For achieving these goals, micronutrient enrichment in the human body is the most important and proven preventive and curative intervention (Wagstaff et al. 2006; Mayer et al. 2008). Although the addition of micronutrient supplements was known to be effective, but due to its high cost and complicated procedure, biofortification

was first conceptualized by the scientists working on biofortification at various CGIAR institutes. It was thought that the results similar to food fortification could be obtained by more oriented breeding for the selected vitamins and minerals into selected staple crops, which should be region specific, as the food habits and the kind of staple foods vary greatly depending on the region (Croft-Cusworth 2018).

The results of a decade of research on biofortification have generated enough proof of concept which has resulted in the approval of the Biofortification Challenge Programme in 2002, which was renamed in 2003 as HarvestPlus (HarvestPlus 2018). This is a part of the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH) and is actually a global partnership program aimed for the development and promotion of biofortified crops (HarvestPlus 2018). HarvestPlus works with an ultimate aim to reduce the hidden hunger among economically weaker sections of the world (HarvestPlus 2016a).

Thus, the concept of biofortification which started taking shape in the mid-1990s actually rechristened into a full-fledged programme as HarvestPlus. Bouis started working for the HarvestPlus as the founding Director and continued on the idea of improving the dietary quality of the staple crops through biofortification of iron, zinc and vitamin A deficiencies, which may lead to blindness, disease or even death, particularly in the children under the age of five (Croft-Cusworth 2018).

Since, 2002 the CGIAR has developed breeding pipelines of seven staple food crops viz., sweet potato, beans, pearl millet, cassava, maize, wheat and rice. The HarvestPlus programme along with International Potato Center (CIP) work on orange-fleshed sweet potato helped in the development and promotion of biofortified crops, especially in the initial phase of biofortification and are still the leading global bodies in the field of biofortification (Croft-Cusworth 2018). On October 13, 2016, Howarth Bouis along with Maria Andrade, Robert Mwanga and Jan Low from the International Potato Center won the World Food Prize for their collaborative work on vitamin A-rich OFSP (orange-fleshed sweet potato). Their work has made an extraordinary dent in the area of biofortification as a novel strategy to fight mineral and vitamin deficiencies, especially in the developing and underdeveloped countries by way of natural enrichment of staple crops (HarvestPlus 2016a; CGIAR 2019).

3 Key Developments in Biofortification of Staple Crops

The biofortification programme which formally came into shape during 1990s started delivering biofortified varieties during the latter half of 2000. Further, from 2014 onwards the release of biofortified crops has been increased and currently more than 300 varieties of 14 crops including bananas, wheat, sorghum, pearl millet, rice, lentil, sweet potato, potato, maize, cassava, beans, etc., which are biofortified with provitamin A, iron and/or zinc have been released in 35 countries and reaching 42.4 million household members in smallholder farming families in low-income countries (Tables 2.1 and 2.2). It is anticipated that biofortified varieties will be available to the farmers in 60 countries within a very short span of time across Africa, Asia and Latin

Table 2.1 List of biofortified varieties developed in different countries with HarvestPlus (HarvestPlus 2014a)

S. no.	Crop	Biofortification for	Target value (ppm)	Country (year)/variety
1.	Banana/ plantain	β-Carotene	17–106	**Ghana**: Apantu **Papua New Guinea**: Bira, To'o **Philippines**: Pelipita **Thailand**: Lai
2.	Bean	Iron	94	**Rwanda** (2010): RWR 2245, 2154, MAC 44, RWV 1129 **Rwanda** (2012): RWV 3006, 3316, 3317, 2887, MAC 42 **DRC** (2008): COD MLB 001, VCB 81013, Hm 21-7, RWR 2245, COD MLV 059 **DRC** (2013): PIGEON VERT, PVA 1438, COD MLB 032, CUARENTINO, NAIN DE KYONDO
3.	Cassava	β-Carotene	15	**Nigeria** (2011): TMS 01/1371, TMS 01/1412, TMS 01/1368 **DRC** (2008): I011661
4.	Cowpea	Iron	63	**India** (2008, 2010, 2013, 2014): Pant Lobia-1, 2, 3, 4
5.	Lentil	Iron	70	**India** (2012): L4704 **Nepal**: ILL 7723, Sisir, Khajurah-1, 2, Khajurah Masuro-4, Sital, Shekhar, Simal **Bangladesh**: Barimasur-4, 5, 6, 7, 8, 9
6.	Maize	β-Carotene	15	**Zambia** (2012): GV662A, GV664A, GV665A **Nigeria** (2012): Ife maizehyb-3, Ife maizehyb-4, Sammaz 38 (OPV), Sammaz 39 (OPV) **Ghana** (2012): CSIR-CRI Honampa (OPV) **China** (2015)
7.	Pearl Millet	Iron	77	**India**: Hybrid #7, Hybrid #12; ICTP 8203-Fe-10-2 (Dhanashakti), ICMH 1201 (Shakti-1201)
8.	Potato	Iron (zinc)	48 (33)	–
9.	Rice	Zinc	28	**Bangladesh** (2013): BRRI dhan 62
10.	Sorghum	Iron (zinc)	60 (32)	–
11.	Sweet potato	β-Carotene	32	**Ghana**: Cri-Bohye **Madagascar**: 199062.1 Cri-Bohye in Ghana, Ejumula, Resisto, Zambezi **Malawi**: Ana Akwanire, Kadyaubwerere, Kaphulira, Mathuthu, Zondeni **Mozambique**: 199062.1 Cri-Bohye in Ghana, Amelia, Bela, Coromex, Cecilia, Cn-1424-9, Cn-1448-49, Delvia, Ejumula, Erica, Esther, Gaba Gaba, Ininda, Irene,

(continued)

Table 2.1 (continued)

S. no.	Crop	Biofortification for	Target value (ppm)	Country (year)/variety
				Jane, JaponTresmesinoSelecto, Jewel, Kandee, Lourdes, Lo-323, Melinda, Namanga, Persistente MGCL01, Resisto, Sumaia, Tainung 64, Tio Joe **Nigeria**: Umuspo/1, Umuspo/3 (Mother's Delight) **Rwanda**: Ejumula, Kakamega Spk004, Rw11-2560, Rw11-2910 **South Africa**: Impilo, Khano, Resisto, W-119, Ejumula, Kakamega Spk004, Kenspot-3, Kenspot-4, Kenspot-5, K566632, W151 **Tanzania**: Carrot C, Ejumula, Kakamega Spk004, Kiegea Kbh2001/261, Matayakbh2001/261, Mayai **Uganda**: Ejumula, Kakamega Spk004, Naspot 8, 10, 12, 13 **Zambia**: Chiwoko, Olympia, Twatasha, Zambezi
12.	Wheat	Zinc	37	**India** (2014): BHU1, 3, 5, 6 (Chitra), 17, 18 **Pakistan** (2015): NR-419, NR-420, NR-421

More details of the varieties are mentioned in Chap. 1

America (Biofortification Strategy 2018). Moreover, this number is constantly increasing by each passing year (HarvestPlus 2019).

Some key developments in the field of food fortification and biofortification of the staple food crops in a chronological order is presented below:

- **1920**: Table salt fortification with iodine in Switzerland and USA.
- **1940s**: Flour fortification in industrial mills.
- **1950–1960**: Dr. Nevin Scrimshaw showed the impact of food supplements on the health of poor children in developing countries.
- **1953**: Some food fortifications were made mandatory in India.
- **1963**: Discovery of opaque-2 (o2) gene in maize by Purdue University researchers.
- **1970**: Nutritional Anaemia Control Programme in India.
- **1970s and 1980s**: Development of QPM by CIMMYT (International Maize and Wheat Improvement Center) as the precursor of biofortification of maize programme.
- **1980s**: 'Hidden hunger' came into limelight.
- **1984**: Agronomic fortification (fertilizers) for selenium has been made mandatory in Finland.

Table 2.2 List of the biofortified varieties released in India in various crops that are enriched for various nutritional contents

S. no.	Crops	Biofortified for	Content	Year	Name of variety/ genotype
1.	Cauliflower	β-Carotene	8.0–10.0 ppm	2015	Pusa Beta Kesari 1
2.	Groundnut	Oleic acid	~80%	2019	Girnar-4 and Girnar-5
3.	Lentil	Iron	65.0 ppm	2017	PusaAgeti Masoor
4.	Lentil	Iron, zinc	73, 51 ppm	2018	IPL 220
5.	Maize	Provitamin-A, lysine, tryptophan	8.15 ppm, 2.67%, 0.74%	2017	Pusa Vivek QPM9 Improved
6.	Maize	Lysine, tryptophan	3.62%, 0.91%	2017	Pusa HM4 Improved
7.	Maize	Lysine, tryptophan	4.18%, 1.06%	2017	Pusa HM8 Improved
8.	Maize	Lysine, tryptophan	2.97%, 0.68%	2017	Pusa HM9 Improved
9.	Mustard	Erucic acid	<2.0%	2013	Pusa Mustard 30
10.	Mustard	Erucic acid, glucosinolates	<2.0%,<30.0 ppm	2016	Pusa Double Zero Mustard 31
11.	Pearl millet	Iron, zinc	73.0, 41.0 ppm	2017	HHB 299
12.	Pearl millet	Iron	73.0 ppm	2017	AHB 1200
13.	Pomegranate	Iron, zinc, vitamin C	5.6–6.1, 0.64–0.69, 19.4–19.8 mg/100 g	2017	Solapur Lal
14.	Rice	Protein	10.3%	2016	CR Dhan 310
15.	Rice	Zinc	22.6 ppm	2016	DRR Dhan 45
16.	Rice	Zinc	25.2 ppm	2018	DRR Dhan 49
17.	Soybean	KTI	KTI-free	2018	NRC-127
18.	Sweet potato	β-Carotene	14.0 mg/100 g	2015	Bhu Sona
19.	Sweet potato	Anthocyanin	90.0 mg/100 g	2017	Bhu Krishna
20.	Wheat	Iron, zinc	40.0, 42.0 ppm	2017	WB 02
21.	Wheat	Iron, zinc	40.0, 40.6 ppm	2017	HPBW 01
22.	Wheat	Protein, iron, zinc	12%, 42.1 ppm, 42.8 ppm	2017	PusaTejas (HI 8759)
23.	Wheat	Protein, iron, zinc	13%, 43 ppm, 35 ppm	2017	PusaUjala (HI 1605)
24.	Wheat	Protein, iron, zinc	14.7%, 46.1 ppm, 40.3 ppm	2018	MACS 4028 (d)

Source: Yadava et al. (2017, 2018), AICRP-G Report (2019)

- **1990**: The World Summit for Children as the landmark event in the fight for hidden hunger.
- **1990s**: Howarth Bouis, Robin Graham and Ross Welch started working on the aspect of biofortification.
- **1993**: Development of 'micronutrient biofortification program'.
- **1994–2002**: Generation of scientific evidence for biofortification under 'CGIAR Micronutrients Project'.
- **1998**: Mandatory table salt fortification with iodine in India to control Goitre.
- **2000**: UN members adopted Millennium Development Goals (MDGs).
- **2000**: Development of 'Golden Rice', the earliest and most prominent biofortified crop.
- **2001**: Steve Beebe, a bean researcher at CIAT coined the term 'biofortification'.
- **2001**: The UN General Assembly adopted eight MDGs resolution of which three are aimed on the nutrition.
- **2002**: Approval of the Biofortification Challenge Programme for biofortification of staples.
- **2002**: Biofortification-based breeding pipelines of seven staple food crops viz. sweet potato, beans, pearl millet, cassava, maize, wheat and rice by CGIAR.
- **2003**: Renaming of Biofortification Challenge Programme as HarvestPlus which started working in Sub-Saharan Africa and South Asia.
- **2003**: IITA has started screening of nearly 2000 cowpea genotypes in Nigeria for the identification of micronutrient-rich genotypes.
- **2003 onwards**: Proof of concept research has been conducted on different target populations for confirming that the biofortification actually works.
- **2004**: Two OFSP cultivars namely, 'Ejumula' and 'Kakamega' were released in Uganda.
- **2004**: ICARDA has initiated research to develop high iron and zinc lentil genotypes.
- **2005**: Beginning of large-scale biofortification program in China for a number of crops and minerals.
- **2006 onwards**: GR2, a transgenic event having up to 37 ppm of 'provitamin A' were backcrossed in the rice varieties of India, Indonesia, Philippines and Bangladesh.
- **2006–2009**: In Uganda and Mozambique, the OFSP intervention impacted 24,000 households.
- **2008**: Pant Lobia-1 a biofortified cowpea variety for Fe and Zn was released from G.B. Pant University of Agriculture and Technology, Pantnagar, India.
- **2009**: Start of multi-nutrient biofortification in different crops.
- **2009**: Copenhagen Consensus ranked biofortification as the highest value-for-money investment.
- **2009–2013**: First wave of biofortified varieties in a number of crops were bred and released. Conduction of nutritional efficacy trials and development of micronutrient delivery plans for the target population.
- **2010**: African Biofortified Sorghum project.

- **2011**: Golden Rice Project, Bio-cassava Plus project, and Better Bananas for Africa project.
- **2012**: HarvestPlus started working in Latin American and the Caribbean.
- **2013–2014**: Efficacy trials for orange maize in Zambia; yellow cassava in Nigeria; beans in Rwanda.
- **2014**: At second International Conference on Nutrition (Rome), the countries like Bangladesh, Malawi, Nigeria, Pakistan and Uganda put emphasis to include the biofortification in their national policies.
- **2014 onwards**: Scaling up of biofortified crops delivery to the most needed populations across the world.
- **2014**: Release of more than 140 biofortified varieties of 10 staple crops in 30 countries.
- **2015**: Adoption of the Sustainable Development Goals (SDGs) by the UN members.
- **2015**: HarvestPlus collaborated with the CIMMYT along with 30 other national and international partners for the development of vitamin A-enriched maize.
- **2016:** Wheat flour fortification with folic acid in 61 countries.
- **2016**: More than 20 million people in 4 million farming households in HarvestPlus target countries grew and consumed biofortified crops.
- **2016**: Howarth Bouis, Maria Andrade, Robert Mwanga and Jan Low won the World Food Prize.
- **2016**: Under the UN Decade of Action on Nutrition, the CGIAR is given the responsibility to lead mass-scale adoption drive of biofortification product at global level.
- **2016**: Setting up of 'WHO Cochrane review committee' for the review of scientific evidence and country experiences of biofortification.
- **2017**: More than 150 biofortified varieties of 10 crops have been released in 30 countries, benefitting nearly 33 million people in 10 million poor farming households.
- **2018:** Brazil, India and China adopted 'food basket' approach for biofortification.
- **2018**: India became the first country to have a minimum level of iron and zinc to be bred into the pearl millet varieties.
- **2019**: More than 300 biofortified varieties of 11 staple crops are released in 35 countries which has reached 42.4 million household members.
- **2019**: The biofortified crops are being grown and tested in more than 60 countries.
- **2030**: Target to reach 1 billion people in both rural and urban settings as the consumer of biofortified foods.

4 Progress of Biofortification Work Across the Globe

Until 2012, HarvestPlus worked in only Sub-Saharan Africa and South Asia, then it started working in Latin American and the Caribbean countries (AgroSalud 2011; Lividini and Fiedler 2015) and is constantly moving ahead in terms of the number of

countries where biofortification work has been initiated and also for the number of varieties released (Saltzman et al. 2012). Other biofortification efforts include the use of genetic engineering-related projects like Golden Rice Project (2011), Bio-cassava Plus (Sayre et al. 2011), African Biofortified Sorghum (ABS 2010) and Better Bananas for Africa (QUT 2011). In addition, there are a number of other specialized projects targeting biofortification in various crops (INSTAPA 2011; BAGELS 2008; HarvestZinc 2011; Lividini and Fiedler 2015) and this number is constantly on the rise across different parts of the world. Breeding targets for the micronutrients are set as per the local consumption levels of staple crops, their retention while preparing the food and bioavailability so as to realize the measurable health impacts (Meenakshi et al. 2007). Accordingly, breeders aim for different concentration of various micronutrients and the vitamins as a maximum attainable level in short to medium term. The details of the target value of various traits as fixed by the HarvestPlus (2014a) are mentioned in Table 2.1.

The number of biofortified crops developed/grown per country is varying from one to many and countries like Brazil, India and China are moving ahead with 'food basket' approach and are targeting a number of crops like wheat, rice, lentil, pearl millet, etc. (Lockyer et al. 2018). Each year, there is an increase in the number of countries accepting biofortification across the globe, and countries like Afghanistan, Eritrea, Chad, Gabon, Gambia, Morocco, Lebanon, South Sudan and Tunisia have also released or tested various biofortified crops. Currently, various biofortified crops like sweet potato, cassava, maize, beans, pearl millet, rice, etc., are cultivated in more than 60 countries across Africa, Asia, and Latin America and is benefitting millions of needy people. Thus, biofortification is considered as one of the most important nutritional interventions at the global level (HarvestPlus 2016a, b). In India, various crop-based institutes which are working under the ambit of ICAR (Indian Council for Agricultural Research) have also developed a number of biofortified varieties of cereals, oilseeds, pulses, vegetables and fruit crops (India Spend 2019) (Table 2.2). Now biofortification is accepted as a proven method for enhancing a number of essential vitamins and minerals to the rural-based poor households that are primarily dependent on staples for their nutrition (Croft-Cusworth 2018). The larger aim of biofortification is to benefit at least a billion people across the world by 2030 (HarvestPlus 2016b).

Most of the biofortification studies are generally aiming for a single micronutrient while the poor population often suffers from multiple nutrient deficiencies. Thus, aiming to develop multiple varieties for different micronutrient may not be a practical way to combat the hidden hunger. Multi-biofortification could be an alternative strategy to fortify a crop with more than one micronutrient. In this regard, Naqvi et al. (2009) developed biofortified maize for provitamin A, vitamin C and folic acid using transgenic approach. Such a strategy is also used for other crops like rice, cassava, sorghum, banana, etc. (Qaim et al. 2007). Steur et al. (2012) also conceptualized the multi-biofortification of rice with provitamin A, zinc and iron and folate. In India, a number of varieties which are multi-biofortified have been released, especially for maize, brassica and pomegranate (Table 2.2).

Compared to Fe and Zn the work on the development of varieties enriched in iodine (I) and selenium (Se) is not that widely attempted (White and Broadley 2009). As per the WHO and USDA recommendation, the required dietary intake of Se in adult humans is 55–200 μg/day (Thomson 2004; WHO 2009) while RDA for iodine is 150 μg/day, which is generally lacking in the normal staple food. A number of research projects have been sanctioned for the biofortification for I (Blasco et al. 2010; Cakmak et al. 2017; Lawson et al. 2015; Li et al. 2017) and Se (Hawrylak-Nowak et al. 2015). Moreover, the interaction between I and Se, which may occur during the plant development is not well known (Lyons 2018). A few studies have confirmed the possibility of simultaneous biofortification of I and Se in the hydroponic system for the crops like spinach (Zhu et al. 2004) and Se + Zn + I enrichment of wheat, maize, soybean, potato, canola and cabbage (Mao et al. 2014). In carrot combined I and Se biofortification is feasible with differential uptake ability of the cultivars and I uptake was less efficient than the Se (Smoleń et al. 2019). However, a lot needs to be done for the micronutrients like iodine and selenium biofortification.

4.1 Agronomic Biofortification

Agronomic biofortification involves the physical application of nutrients so as to enrich the crop with the selected nutrients which in turn improves the nutritional status of the humans (Cakmak and Kutman 2017). When compared with the inorganic form, the organic form of minerals is more available for human body as they can be easily absorbed (Daniels 1996). Among various micronutrients, Zn and Se are found most effective for the agronomic biofortification (Cakmak 2014). To tackle the problem of low selenium in the soil, agronomic fortification for selenium has been made mandatory in Finland since 1984 by the addition of inorganic selenium into all the fertilizer. This has resulted in significantly increased gain Se (Alfthan et al. 2015) which in turn resulted in a significant reduction in the Se deficiencies among the population (de Valença et al. 2017).

The feasibility of selenium biofortification in wheat has also been shown in UK (Broadley et al. 2010) and New Zealand (Curtin et al. 2008) through agronomic fortification. In soybean, the selenium enrichment has been performed successfully through foliar application fertilizers having selenium complex salts (Yang et al. 2003). For chickpea, plant growth-promoting actinobacteria was used for the enrichment of Fe, Zn, Ca, Cu, Mn and Mg (Sathya et al. 2013), while AMF was used for the enrichment of Fe and Zn (Pellegrino and Bedini 2014), and foliar spray was used for the fortification of Zn and Se (Shivay et al. 2015; Poblaciones et al. 2014). In field peas, the Zn fortification was done through foliar application of Zn alone and also in combination with soil application of Zn (Poblaciones and Rengel 2016). Beans have been enriched with zinc by the application of foliar zinc fertilizer (Ibrahim and Ramadan 2015). The list is quite exhaustive and this method is being used mainly for the biofortification studies. Depending on the success of different methods tested and the cost economies, there is a need to use the best combination at the commercial scale so that the benefit of biofortification can reach the resource deprived and the

needy population of the world. Finer details of the agronomic biofortification are dealt in Chap. 1.

4.2 Molecular Markers and Transgenic Technology for Crop Biofortification

Besides breeding-based micronutrient enrichment of the staples, now a number of molecular markers are also identified for the precise and quick development of biofortified varieties (Mishra and Singh 2015; Mishra et al. 2009). In crops like lentils (Khazaei et al. 2017a; Kumar et al. 2019a; Singh et al. 2017, 2019), chickpea (Upadhyaya et al. 2016), faba bean (Baloch et al. 2014), *Phaseolus vulgaris* (Cichy et al. 2009; Blair et al. 2009), *Medicago truncatula* (Sankaran et al. 2009), *Lotus japonicus* (Klein and Grusak 2009) and a number of other crops, various molecular markers have been used to identify the genes/QTLs associated with the micronutrients like iron and zinc contents in seeds. The work on the enrichment of groundnut for oleic acid is being done across the globe using various molecular tools like CAPS and AS-PCR markers (Nawade et al. 2018). India is also working on the development of high oleic (HO) lines in the groundnut, and a number of HO genotypes are identified using these allele-specific DNA-based markers (Nawade et al. 2016, 2019; Janila et al. 2016). In the year 2019, Directorate of Groundnut Research (Junagadh, Gujarat, India) in collaboration with the ICRISAT (Patancheru, India) has developed two high oleic (nearly 80%) groundnut varieties namely Girnar-4 and Girnar-5 in India (AICRP-G Report 2019).

In addition, the transgenic approach has also been exploited to increase its mineral accumulation ability mainly of iron and zinc through increased uptake from the soil (Kerkeb et al. 2008), and reduction in the anti-nutritional factors content like phytic acid (Kumar et al. 2019a). For the delivery of provitamin A in rice, the carotenoid biosynthetic pathway has been reconstituted in the non-carotenogenic endosperm of the rice as Golden rice using phytoene synthase (*PSY*), phytoene desaturase (*CrtI*) and lycopene β-cyclase (*β-lcy*) genes (Al-Babili and Beyer 2005; Schaub et al. 2005; Paine et al. 2005; Ye et al. 2000; Beyer et al. 2002). Transgenics enriched in vitamin has been developed in a number of crops like wheat using *psy1*, *crtI*, *CrtB* or *CrtI* (Cong et al. 2009; Wang et al. 2014), maize using *crtB*, *crtI*, *psy1* genes (Aluru et al. 2008; Naqvi et al. 2009), cassava using *crtB* gene (Welsch et al. 2010) and canola using *crtB* and *crtI* genes (Ravanello et al. 2003).

Similar metabolic pathway reconstitution was also done in other crops like canola, tomato and potato (Shewmaker et al. 1999; Romer et al. 2000; Ronen et al. 2000; Rosati et al. 2000; Diretto et al. 2007). Transgenic for biofortified Zn in rice has been developed using *HvNAS1*, phytase, and *OsNAS1* genes (Masuda et al. 2009; Wirth et al. 2009; Johnson et al. 2011). Transgenic approaches are also used in other breeding programmes like the development of tomato with 15-fold higher folate accumulation (Diaz de la Garza et al. 2007), rice with two-fold more iron (Storozhenko et al. 2007; Lucca et al. 2001; Goto et al. 1999; Masuda et al.

2012, 2013; Johnson et al. 2011) and wheat with high Fe by the introduction of various ferritin genes (Drakakaki et al. 2000; Borg et al. 2012).

The soybean was used to increase provitamin A, oleic acid and seed protein contents (Schmidt et al. 2015). The provitamin A was increased using bacterial PSY genes (crtB, crtW, bkt1) (Pierce et al. 2015), methionine content was increased using cystathionine γ-synthase gene (Song et al. 2013), while α-linolenic acids could be reduced by silencing of ω-3 FAD3 gene (Flores et al. 2018). In addition, antisense RNA technology has been used to increase the amount of oleic acid by inhibiting the expression of FAD gene. The transgenic soybean varieties rich in oleic acid and linoleic acid were released in the European Union, Mexico, South Korea and Taiwan (Garg et al. 2018). In common bean, methionine content was enhanced by expressing the methionine-rich storage albumin from Brazil nut (Aragao et al. 1999). Lupine seed protein methionine content has been enhanced by the expression of sunflower seed albumin gene (Molvig et al. 1997). Recent advancements in the 'omics' technologies, genome editing tools such as transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 and genome sequencing in a number of staple crops have helped in the more precise and quick development of biofortified products (Ricroch et al. 2017).

5 Some Key Crop-Based Biofortification and Their Impact

5.1 Sweet Potato (*Ipomoea batatas*)

Sweet potato is one of the staple foods in the sub-Saharan Africa (SSA) and OFSP is having very high β-carotene content. For the improvement of African sweet potato germplasm, high β-carotene South American sweet potato germplasm is being used for the development of biofortified varieties (Van Jaarsveld et al. 2005). The first OFSP variety was released in 2003, while more than 42 OFSP varieties were bred based on the requirement of farmers and the consumers (Lowa et al. 2017). During 2000, in Mozambique, World Vision and HarvestPlus started assisting plant breeders to test new OFSP varieties (World vision 2020). During 2013 a project named 'Scaling Up Sweet potato through Agriculture and Nutrition (SUSTAIN)' and was led by CIP along with more than 20 partners was launched in Kenya, Malawi, Mozambique, Rwanda, Bangladesh and Tanzania under which farming families were given sweet potato cuttings and as a result more than 2 million households in those countries have improved their vitamin A deficiency (CIMMYT 2019b). Two years of proof-of-concept study in Mozambique has showed a significantly higher vitamin A among young children of the household that grew and consumed the OFSP and the reduction in the rates of vitamin A deficiency was nearly 15% (CGIAR 2019). Interestingly, only 125 g of fresh OFSP provides the daily vitamin A needs of a pre-school child, and simultaneously it also provides high levels of vitamins B6 and C, Mn and K (CIMMYT 2019b).

5.2 Maize (*Zea mays*)

The vitamin A-enriched maize developed by CIMMYT, International Institute of Tropical Agriculture (IITA) and ICAR are available in 19 countries of Africa, Asia, and Latin America (CIMMYT 2019a). The work was initiated in the late 1990s and supported by various research organizations and other partners. Since 2015, HarvestPlus has collaborated with the CIMMYT, Department of Research and Specialist Services, and more than 30 national and international partners, in breeding vitamin A enriched orange maize. Under this programme, the HarvestPlus/Zimbabwe has distributed 64 tonnes of vitamin A maize seed during 2017–2019 to nearly 42,000 household and by December 2018, it reached more than 250,000 households, while by 2020, an anticipated 400,000 smallholder farmers will be growing biofortified crops (HarvestPlus 2019). To secure the long-term sustainability and competitiveness, seed companies are also engaged in the seed multiplication of vitamin A maize, so as to establish their own product lines (Allafrica 2019).

Another refugee-focused project by HarvestPlus is underway in Uganda which is in collaboration with Self-Help Africa, where it supports more than 1000 households during the year 2019–20. In addition, HarvestPlus Zambia is also assisting the orange maize business model for the farmers based on developing linkages with input suppliers and establishing contracts with the purchasers. The objective is to help farmers to self-sustain their investment in orange maize cultivation after receiving start-up support during 2019–2020 (IPS News 2020). CIMMYT, HarvestPlus and Semilla Nueva are working together for the development and deployment of the world's first biofortified zinc-enriched maize hybrid, ICTA HB-18in Guatemala which was released in May 2018. This hybrid contains 6–12 ppm more zinc and 2.5 times more quality protein compared to conventional maize varieties (CIMMYT 2018).

5.3 Wheat (*Triticum aestivum*)

Bioavailability of zinc in Zn-wheat is demonstrated by Rosado et al. (2009) and DNA strand breaks are found as a sensitive indicator of increase in zinc intake from a biofortified crop (King et al. 2016). In India, the development and distribution of zinc wheat varieties was done through ICAR, State Agricultural Universities (SAUs), CIMMYT, seed companies, and non-governmental organizations (NGOs) (Poshan 2020). Six biofortified wheat varieties were released in India and Pakistan with 6–12 ppm more zinc than that of traditional wheat varieties (CIMMYT 2019a). Zincol 2015, a high-zinc and high-iron variety, has benefitted more than 200 million in Pakistan (World vision 2020). In late 2019, large number of farmers were briefed in Bihar State (India) about two newly developed zinc-wheat varieties. It is expected that the zinc-wheat will reach more than 1 million farming households in Bihar in the next 5 years which is expected to cause better health impacts in a state where zinc deficiency contributes to India's highest rates of stunting (Poshan 2020).

5.4 Rice (*Oryza sativa*)

In the year 2013, the first Zn rice variety was released in Bangladesh (Chowdhury 2014) which has the 30% higher Zn content over local varieties (HarvestPlus 2014b). Interestingly the majority of the Zn was concentrated in the endosperm and not in the periphery of the grain, which gets during rice polishing. It is speculated that if this variety is consumed in countries like Bangladesh, where the large amount is being consumed by the poor, it can substantially meet the daily Zn requirement. Since rice is consumed in large quantity by the poor in Asia and Africa, this was chosen as a target for the improvement of betacarotene and iron content through a transgenic approach.

5.5 Barley (*Hordeum vulgare*)

The approval by the Food and Drug Administration (FDA) of the USA to the claims that the soluble β-Glucan in barley is having various health benefit has suddenly increased the interest of the consumer's in the use of barley-based products (CGIAR 2018). Like many crops, ICARDA also has the global mandate for barley RandD and is working in strong association with National Agricultural Research Systems (NARS) and various other institutes (CGIAR 2018). ICARDA is also working with Small Grain and Potato Research Center (Aberdeen Idaho, USA), which has released barley varieties namely, 'Julie' and 'Transit', having high β-Glucan contents. ARS, USDA Aberdeen has also developed nearly 15% higher β-Glucan lines through mutation breeding approach. Materials Transfer Agreement (MTA) has been signed between ICARDA and USDA to use their technology for the incorporation of high β-Glucan into ICARDA's germplasm (CGIAR 2018) and work is under progress. The research in barley is aiming to combine high β-Glucan with high Fe and Zn content along with superior bread-making qualities into improved barley germplasm at ICARDA (CGIAR 2018).

5.6 Pearl Millet (*Pennisetum glaucum*)

For pearl millet, in the year 2018, the All India Coordinated Research Project on Pearl Millet (AICRP on Pearl Millet) has fixed a minimum level of iron and zinc to be bred into the varieties, making India the first country to have such standards for millet varieties. A study has revealed that the introduction of iron-pearl millet in the diet of adolescents in India (Finkelstein et al. 2015) has resulted in reduced iron deficiency and improved learning skills and mental ability (Scott et al. 2014; India Spend 2019).

5.7 Cassava (*Manihot esculenta*)

Yellow or golden cassava are the cassava varieties with high content of β-carotene which was released in the year 2013 in Nigeria where nearly 100 million eat this crop daily. A study has shown significant improvement in vit. A level of the children consuming yellow cassava (Talsma 2014). More than 500,000 farmers are now planting biofortified cassava (HarvestPlus 2014c).

5.8 Beans (*Phaseolus vulgaris*)

Beans are essentially consumed by nearly 400 people in the tropics and iron beans are reaching the plates of African population. During 1994–2002, CIAT has identified the genotypes having iron in the range of 30–110 ppm while zinc as 25–60 ppm. In Rwanda, HarvestPlus worked in association with Rwanda Agriculture Board for the production of biofortified bean iron, through contracted farmers, cooperatives and small seed companies (Asare-Marfo et al. 2016). A study by CIAT in Rwanda has revealed a reversal of iron deficiency symptoms in the young women who consumed iron beans daily for a period of 4.5 months (Haas et al. 2016). Similarly, iron-bean consumption was found effective in primary school children in Mexico (Haas 2014) in terms of improved memory and better cognition ability (Murray-Kolb et al. 2017; Luna et al. 2015).

5.9 Faba Bean (*Vicia faba* L.)

Faba bean has specific significance to food, nutrition and income security in many countries of North and East Africa (Biofortification Strategy 2018). For the improvement of faba bean, ICARDA has the world mandate from CGIAR, and is collaborating with more than 32 countries (CGIAR 2018). The study on genetic variability of only 129 Turkish accessions on microelements (Baloch et al. 2014) has shown a wide range of variability for Fe (29.7–96.3 mg/kg), Mn (15.5–29.2 mg/kg), Cu (10.3–33.0 mg/kg) and Zn (10.4–49.3 mg/kg), indicating the possibility of biofortification. Khazaei et al. (2017b) reported a high throughput low-cost KASP marker for low vicine and convicine (v-c) concentration.

5.10 Lentil (*Lens culinaris*)

Lentil is a staple crop in many developing countries like Ethiopia, Bangladesh, India and Nepal where hidden hunger is also prevailing. The screening of more than 2200 lentil genotypes recorded the range for Fe content as 41–168 ppm while Zn content in the seed was found ranging between 22 and 103.7 ppm (CGIAR 2018). Since 2004, International Center for Agricultural Research in the Dry Areas (ICARDA) has initiated research to develop high iron and zinc lentil genotypes, which has

resulted in the release of a number of biofortified varieties in Bangladesh, India, and Nepal (HarvestPlus 2014a) which are under cultivation in the targeted areas of the country.

5.11 Cowpea (*Vigna unguiculata*)

During 2003–2008, IITA has started screening of more than 2000 cowpea lines in Nigeria and the biofortification of cowpea programme was later moved to G.B. Pant University of Agriculture and Technology, Pantnagar, India. Two early-maturing high-iron and zinc cowpea varieties, Pant Lobia-1 and Pant Lobia-2, were released by the Uttarakhand Government in 2008 and 2010, respectively. Further, Pant Lobia-3 and 4 were released in 2013 and 2014, respectively. These varieties have now entered the national seed multiplication system and seed is available to farmers.

6 Reasons of Success and Challenges for the Adoption of Enriched Crops

It is almost three decades since when biofortification work has started taking shape and during this period various key factors leading to the success of crop biofortification have been identified. On the ground, HarvestPlus, CIP and various CGIAR centres are working with more than 500 partners across the globe, including different national governments, research institutions, NGOs and farmers (Croft-Cusworth 2018). Another reason for the success of the biofortification programme is quick generation of evidences in the support of micronutrient enrichment, along with monitoring and evaluation. In most of the biofortification projects, scientists focused on not only product development but also on the dissemination of products and impact assessment (Croft-Cusworth 2018). Another key factor for the success is the maintenance of a clear research vision and its anticipated impact on the target population. Thus, research was aimed beyond the laboratory and inclusion of farmers and consumers preference are taken care even at the very beginning of the project. Finally, keeping track of the investment needed for both research and dissemination, focused work on partnerships, monitoring and evaluation, and pathways to impact all have helped in the success of the biofortification projects as we see it today (Croft-Cusworth 2018).

However, product cost and regulatory compliance appears major constraint in the development of nutrient-enriched crops, more so when biotechnological interventions are involved (Powell 2007). In addition, the transgenic technology tends to be proprietary and involves IPR issues which needs due care. A successful enrichment should be followed with the widespread adoption of the biofortified product by both farmers and consumers (Powell 2007). In addition, if a trait changes the appearance, taste, etc. of the product then the problem of public acceptance appears as observed for the golden rice. To overcome this, adequate information should be generated before bringing the product in the market. Lack of agricultural

infrastructure in a number of developing countries poses a severe challenge for the adoption of biofortified varieties.

7 Conclusions and Future Prospects

The global pressure of micronutrient deficiency came down by half between the year 1990 and 2010 (Wang et al. 2012). Among vit. A, iron and zinc deficiencies, the maximum disease reductions were recorded for vit. A deficiency. Nonetheless, micronutrient deficiencies are still the key public health concern especially in Sub-Saharan Africa (Lim et al. 2012). In coming decades biofortification should be more intensively tied up for meeting the two global sustainable development goals (SDGs) viz. 'end hunger, achieve food security and improved nutrition and promote sustainable agriculture' (SDG2) and 'ensure healthy lives and promote well-being for all at all ages' (SDG3), and also simultaneously managing the climate change (Croft-Cusworth 2018; UNSDG 2016). Public sector should play a vital role, by way of suitable policies and programs favouring the cultivation and consumption of biofortified crops across the world by the neediest populations so as to have the panacea for the deadly 'hidden hunger' (Poshan 2020).

An IFPRI study has identified the possibility of introducing the biofortified wheat and rice through the state-run public distribution system (PDS), aiming to reach the economically weaker section at an affordable price especially in the most affected states of India viz. Bihar and Odisha (IFPRI 2019). If the normal rice which is distributed through PDS in these states is replaced by Zn rice then the estimated increase in the Zn intake would be by 60%, while biofortified wheat for Fe and Zn may increase its intake by more than 30% (IFPRI 2019). A study was performed in Maharashtra state (India) on the adolescent-age poor students, wherein they displayed significantly better learning capabilities after consuming the foods enriched with the Fe pearl millet for a period of 6 months. Similarly, another 6-month study in Delhi state (India) involving children (4–6 years) and their mothers when fed with zinc-wheat based foods showed better health in terms of illness (Poshan 2020). Additional studies are needed to reveal more positive effects of biofortified staple crops on nutrition (Listman et al. 2019).

Inclusion of biofortified pearl millet, rice and wheat in the Indian PDS would surely improve the nutrition composition of the food which is enriched in the most desired micronutrients to a larger section of the underprivileged population. The Bihar state of India has developed a 'Bihar Agriculture Road Map 2017–2022' so as to make available the biofortified crops to the neediest (Poshan 2020). However, the introduction of biofortified crops through PDS without any rigid physical and institutional infrastructure for its production, procurement and distribution might not work. For the sustained success of the biofortification programme from the development of the variety till it reaches the plate of the needy, incentivization of the farmers by providing them a premium price of the biofortified produce seems inevitable for quick penetration of these varieties (Poshan 2020).

Strong systems for sampling, testing, grading and marking will be essential to maintain the traceability of produce. Later, similar possibilities could also be explored for biofortified pulses and oilseeds (IFPRI 2019). In addition, the varieties developed by any country should be amalgamated as a core product in their research, policy and food value chains so that all the stakeholders of the value chain, especially the farmers and consumers, must be convinced of their value (Listman et al. 2019).

Another initiative of including the biofortified crops was taken by the Women and Child Development Ministry (Government of India) under the Poshan Abhiyan (National Nutrition Mission) so as to encourage the dietary diversity at the community level (Poshan 2020). As per the Biofortification Priority Index (BPI) of HarvestPlus, which ranked the 128 countries for the investment's potentials of eight biofortified staple food crops, India ranked among top 10 countries in terms of the benefit from investment in iron pearl millet, while it ranked third for zinc wheat. In case of OFSP in Uganda, the ex-post cost-effective data showed USD 15–20 per Disability Adjusted Life Year (DALY) through biofortification which is considered highly cost-effective by World Bank (World Bank 1993; HarvestPlus 2010). The Copenhagen Consensus also ranked biofortification as the highest value-for-money investment and for 1 USD invested the benefit that could be gained is to the tune of 17 USD (Hoddinott et al. 2012; Meenakshi 2009).

Inclusion of biofortified pearl millet, rice and wheat in the mid-day meal scheme can have the potential to improve the health of several million children. There is a need that the government of different countries should aim to 'make every farm a biofortified farm' for the most efficient and sustainable way of delivering the essential micronutrients to the neediest smallholder farming families (Poshan 2020).

Biofortified crops are aimed to complement the existing micronutrient deficits and in turn it should have visible impact on the health of millions of people suffering from hidden hunger (Pocket 2007). The use of biofortification strategy for the improvement of micronutrient contents of the staple crops across the world needs strong support from different agencies (Biofortification Strategy 2018). This needs focused efforts beyond the scientific community (Rehman et al. 2018) and investments should be poured for both scientific and economic research so that biofortified crops can be developed as per the need of the region and accordingly introduced to those areas. To achieve the target of 1 billion people by 2030, biofortification must expand itself beyond a few target-oriented projects. Policymakers should consider biofortification as a means of improving the human health and it should be made as a part of government's nutrition agenda. The breeding unit of both public and private sector must focus on the biofortification across their product lines (Birol and Bouis 2019). A necessity should be felt and demand should also come from the consumers for the biofortified products. Thus, only by way of collective efforts spanning across the value chain, the vision of reaching 1 billion with biofortified products can be achieved.

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Genetic Fortification of Rice to Address Hidden Hunger: Progress and Prospects

3

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Abstract

Micronutrient malnutrition or hidden hunger is affecting more than 2 billion people globally. The nutrient deficiency is recognised as a major challenge in achieving the United Nation's Sustainable Development Goals. Biofortification aims at enhancing the micronutrient status of the staple foods through genetic means. Breeding for improved varieties having nutrient enriched grains is a targeted, sustainable and cost-effective approach to alleviate the hidden hunger. Currently, rice is identified as the choice crop for biofortification as it feeds more than half of the global population. This chapter provides a comprehensive review on the progress and prospects of biofortification in rice, with specific emphasis on Fe, Zn and pro-vitamin A carotenoids. Globally, significant progress has been made in surveying the rice germplasm for the micronutrients, and identified several QTLs governing their accumulation, uptake, translocation and storage in the rice grain. However, relatively less progress have been made in molecular breeding of rice towards nutrient enrichment. Recent advancements such as genetic engineering and genome editing provide future promise in the biofortification programmes. Bio-availability rather than quantity should be considered as the determining factor for nutrient enrichment. A holistic approach is required to include stable donors for future varietal development, targeting rice biofortification and consequent alleviation of hidden hunger.

Keywords

Iron · Zinc · Pro-vitamin A · Uptake · Translocation · Storage · Bioavailability · Molecular mapping · QTLs

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1 Introduction

Micronutrients, the minerals and vitamins that are required in minuscule amounts play key roles for maintaining proper human physical and mental health. They include potassium (K), chloride (Cl), sodium (Na), calcium (Ca), phosphorus (P), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), iodine (I), chromium (Cr), molybdenum (Mb), selenium (Se) and cobalt (Co) and vitamins such as A, B, C, D and E. Long-term insufficiency of these essential micronutrients can impair growth and development, a situation called micronutrient malnutrition or hidden hunger. Globally, deficiencies of Fe, Zn and vitamin A are more prevalent, affecting a large segment of human population. According to an estimate, more than half of the global population is suffering from one or the other form of micronutrient malnutrition today. The situation is grim in developing nations of South-East Asia and Africa where access to diversified diets is often limited (Fig. 3.1). Nonetheless, malnutrition is not uncommon in the affluent countries too, but due to unbalanced diets.

Among the micronutrients, Fe plays a prominent role in maintaining the human health. It helps in the oxygen transport through the synthesis of oxygen carrier proteins, such as haemoglobin and myoglobin. About 85% of the total Fe in the human body is present in the haemoglobin, which is used as a biomarker for assessing Fe deficiency. The remaining 15% is present as a constituent of myoglobin in the muscle tissue as well as in other enzymes such as cytochromes regulating electron transfer and oxidative metabolism during respiration. A meagre amount of Fe is contained in non-enzyme compounds (Hurrell 1997). Fe is stored in the liver as ferritin, and transported across the body as a component of a protein called transferrin (McDowell 2003). Fe deficiency is the most common form of micronutrient malnutrition affecting more than 30% of the world population (De Benoist et al. 2008). Deficiency is indicated when the serum ferritin falls below 30 mcg/L while a value below 10 mcg/L specifies Fe deficiency anaemia (IDA) (Camaschella 2015). IDA occurs on exposure to long-term deficiency of Fe, which bears implications on reduced work capacity and productivity of individuals. Pregnant women have a higher requirement of Fe owing to foetal growth and development and a deficiency during pregnancy may lead to complications in childbirth including premature childbirth, low birth weight and other perinatal complexities (Bailey et al. 2015). According to an estimate of the World Health Organization (WHO), almost 20% of maternal deaths are ascribed to IDA alone (Bailey et al. 2015) and globally about 40% of the pregnant women and 42% of the children are anaemic (Stevens et al. 2013). Fe is particularly critical in early life and has substantial influence in determining the human capabilities at the individual level by regulating physical and cognitive development (Lozoff et al. 2013).

Similar to Fe, Zn is essential to all living organisms including humans as a metabolic regulator. Zn is the only metal element which is integral to the enzymes of all six major classes viz. oxidoreductases, hydrolases, transferases, lyases, ligases and isomerases. Involved in catalytic, structural and regulatory roles (Vallee and Auld 1992; Coleman 1992), Zn is also associated with nucleic acid metabolism

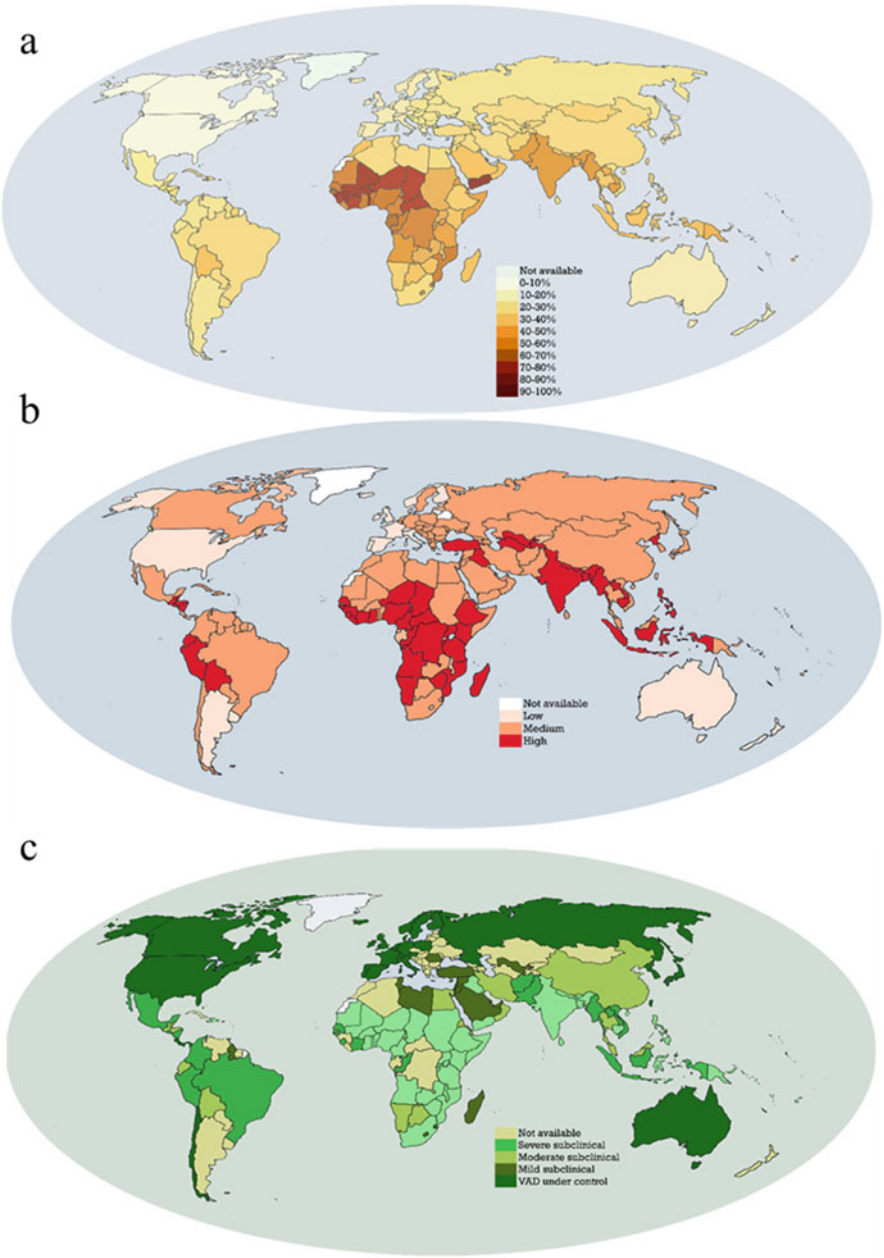

Fig. 3.1 Global distribution of severity of Fe (**a**), Zn (**b**) and pro-vitamin A (**c**) deficiencies

being intrinsic to DNA and RNA polymerases, Zn-finger proteins, reverse transcriptases and transcription factors (Wu and Wu 1987; Coleman 1998). Additionally, the role of Zn has also been reported in the maintenance of membrane stability and integrity, moderation of oxidative stress and transiting extracellular stimuli into intracellular signalling pathways as an intracellular second messenger (Cakmak 2000; Disante et al. 2010; Yamasaki et al. 2007). The importance of Zn in human health and nutrition is evident from the fact that the human body contains approximately 3000 Zn-binding proteins (Andreini et al. 2006). Zn deficiency is often associated with stunting in children as it plays a major role in cell division, growth and carbohydrate metabolism (Sanna et al. 2018). Moreover, Zn deficiency leads to a weakened immune system leading to increased susceptibility to infectious diseases like pneumonia, diarrhoea and malaria (Patel et al. 2010; Black 2003). Furthermore, it also leads to learning disabilities in children, neural atrophy and impaired memory (IZiNCG et al. 2004). An estimated 17.3% of the global population has insufficient dietary Zn intake, with Africa having the highest proportion (23.9%) followed by Asia (19.4%) (Bailey et al. 2015). According to the recent survey conducted by UNICEF, approximately 149 million children under the age of 5 are affected by stunting. Zn deficiency also increases childhood morbidity and mortality causing about 800,000 early age deaths every year (Caulfield et al. 2006).

Among the vitamins, the deficiency of vitamin A, a fat-soluble vitamin, is a widespread and major public health concern in the developing world especially in Sub-Saharan Africa and Asia. Vitamin A is essential for proper vision and immune system development. Vitamin A deficiency (VAD) often leads to a condition called xerophthalmia (dry eyes) with impaired vision, while a severe deficiency can cause complete blindness. Moreover, VAD weakens the immune system predisposing individuals to infectious diseases like diarrhoea and measles and ultimately the risk of death (Scrimshaw and SanGiovanni 1997; Christian and West 1998). According to the estimates of WHO, VAD has resulted in blindness in about 250–500 children globally.

Cereal grains form the major source of energy and mineral supply in the human diet. However, only few of them adorn the status of staple cereals, such as rice, wheat, and corn. Naturally, staple cereals are marginal sources of essential micronutrients. Therefore, a large-scale reliance on cereal-based food has been identified as the root cause of micronutrient malnutrition. Although micronutrient supplementation to combat hidden hunger has been recommended, it remains unaffordable to many governments (Meenakshi et al. 2010). Alternatively, industrial fortification by post-harvest augmentation of micronutrients in the processed foods is recommended, but can lead to cost escalation of the food items making them pricey to poor and low-income populations (Mayer et al. 2008). Further, industrial fortification of foods often leads to undesirable changes in the colour and flavour of the foods which consequently affects consumer acceptance (Hurrell 2002; Abbaspour et al. 2014). On the other hand, biofortification, a process of enriching the micronutrient status of staple food crops through conventional and molecular plant breeding approaches offers a sustainable solution to address the global micronutrient malnutrition as well as to avert price rise and quality deterioration issues of the industrially

fortified food. Once developed, biofortified crops would be easily accessible to the rural underserved population and also become cost-effective on the long run (Bouis and Saltzman 2017).

Among the staple cereals, rice alone feeds more than half of the global population. Cultivated in more than a hundred countries and occupying approximately 158 million hectares of total harvested area, rice yields more than 700 million tons of paddy equivalent to 470 million tons of milled grains annually. Asia accounts for almost 90% of global rice production equivalent to about 640 million tons. With the increasing human population, the global demand for rice also shows a parallel increasing trend with the total consumption reaching 486.62 million tons by the year 2019 (www.statista.com). The average per capita rice consumption has reached an all-time high of 79.7 kg in the year 2017 (www.helgilibrary.com). Among the countries, Laos stood at the first position with a highest per capita consumption of 271 kg while Poland occupied the last position with 1.61 kg (www.helgilibrary.com). Among the major rice consuming nations, Bangladesh, Cambodia, Vietnam, Indonesia, Philippines, Myanmar, Thailand, Nepal and China stand at the top with an annual per capita consumption exceeding 100 kg. However, the latest statistic from India shows a per capita rice consumption of 103 kg in the year 2017. This enormous reliance on rice, particularly among Asian nations, makes it a candidate staple cereal for biofortification. Ex-ante studies predicted that biofortification of rice could alleviate the burden of micronutrient deficiencies by 19% for Fe, 16% for Zn and 9% for vitamin A, even under mediocre assumptions, at a minimal cost of less than US$ 20 per disability-adjusted life year (DALY) saved (Qaim et al. 2007).

In the last two decades, consistent efforts are in place to enhance the micronutrient status of popular high yielding rice varieties particularly mega varieties through conventional as well as molecular breeding approaches. These works have led to the molecular dissection of the micronutrient content related traits by the identification of a large number of QTLs across various genetic backgrounds. Further, physiological and biochemical basis of uptake, translocation, homeostasis and storage of Fe and Zn as well as biosynthesis of provitamin A have been elucidated using various functional genomic approaches including both forward and reverse genetic tools. With the contemporary advancements in next-generation sequencing technologies, genomics aided crop improvement is on the cards, integrating the application of tools such as marker aided selection (MAS), genome-wide association studies (GWAS) and genomic selection (GS). This would not only guarantee speed and accuracy of biofortification programmes, but also ensure the development of a next series of biofortified and healthy cultivars with several consumer preference options. Since a consolidated compilation of these developments is still lacking, we have summarized the recent advancements in the development of biofortified rice for Fe, Zn and provitamin A in this chapter.

2 Fe and Zn: Natural Variation and Inheritance

Genetic improvement through breeding relies on the existence of significant variability in the crop gene pool including wild congeners. Rice diversity in the world is enormous, occupying various adaptation zones and spread across different cultivated species such as *Oryza sativa* and *O. glaberrima*. Further, *O. sativa* has two major sub-species *indica* and *japonica*, with several intermediary admixtures. With an additional 22 wild species, rice germplasm shows spectacular variability for agronomic characters. However, the micronutrient diversity of rice gene pool is seldom investigated. This necessitates screening of a large number of germplasm accessions, elite varieties, breeding lines and wild species for the traits related to micronutrient accumulation. Wide variation for Fe concentration in brown rice ranging from 0.25 (Roy and Sharma 2014) to 100.45 ppm (Jahan et al. 2013) has been reported in different studies across the world (Table 3.1). However, as the maximum proportion of Fe is localized in outer aleuronic layers and embryo of the rice grain, 90% of it is lost during the process of milling (Bollinedi et al. 2020a). Consequently, a narrow range of 0.7 (Descalsota-Empleo et al. 2019) to 16.9 ppm (Islam et al. 2020) of Fe has been observed in polished rice.

The grain Zn content ranges from 0.85 to 195.3 ppm (Roy and Sharma 2014) in brown rice and 7.43 (Pradhan et al. 2020) to 40.9 ppm (Bollinedi et al. 2020a) in polished rice. This is considerably broader than the Fe content suggesting the scope for improvement through breeding. Of the subspecies, *japonica* accessions were found to accumulate a higher concentration of Zn compared to the *indica* types (Yang et al. 2018; Tan et al. 2019). Aromatic accessions had relatively higher mineral concentration compared to non-aromatic types (Graham et al. 1999). Wild species are valuable resources offering greater potential for bio-fortification. Anuradha et al. (2012) reported a highest Fe concentration of 72 ppm in the brown rice of *Oryza nivara* while Maganti et al. (2020) reported 13.1–22.6 ppm of Fe in the accessions of *O. barthi*, *O. glaberrima*, *O. nivara* and *O. officianalis.* Ishikawa et al. (2017) evaluated a set of wild and cultivated species for grain Zn in brown rice and reported a significantly higher concentration in the Australian wild accession *O. meridionalis* W1627 followed by the *O. glumaepatula* accession W1169.

3 Classical Genetics and Breeding

The genetic basis of the inheritance of grain Fe and Zn in brown/polished rice is very complex and a better understanding of the genetic basis of high grain micronutrient content in rice is essential for the systematic utilization of rice germplasm in mineral biofortification programs.

Several studies indicated a significant positive correlation between grain Fe and Zn contents suggesting the scope for their simultaneous improvement (Stangoulis et al. 2007; Anuradha et al. 2012). Bollinedi et al. (2020a) found poor correlation between Fe concentrations in brown rice and polished rice. Duplicate gene interaction for the inheritance of Fe has been suggested by Samak et al. (2011) based on

Table 3.1 Variation for Fe and Zn in brown and polished rice reported by various studies

Study	No. of accessions analysed	Iron (μg/g)		Zinc (μg/g)	
		Brown	Polished	Brown	Polished
Gregorio et al. (2000)	1138	6.3–24.4	–	13.5–58.4	–
Anuradha et al. (2012)	126	6.2–71.6	–	26.2–67.3	–
Jahan et al. (2013)	52	1.32–100.45	–	–	–
Kumar et al. (2014)	20	9.6–44.0		9.9–39.4	
Bollinedi et al. (2020a, b)	190	6.5–23.1	0.8–12.3	13–46.2	8.2–40.9
Islam et al. (2020)	113	–	1.10–16.90	–	13.33–21.66
Prom-u-thai et al. (2007)	44	10–20	3–11	–	–
Roy and Sharma (2014)	84	0.25–34.8	–	0.85–195.3	–
Kampuang et al. (2017)	12	7–23	–	10–36	–
Nachimuthu et al. (2014)	192	6.6 – 16.7	–	7.1–32.4	–
Yang et al. (2018)	529	69.33–94.49	–	0.27–28.03	–
Zhang et al. (2018a)	698	–	0.9–9.1	–	5.8–29.6
Descalsota et al. (2018)	144	–	1.0–6.9	–	8.1–32.6
Descalsota-Empleo et al. (2019)	156	–	0.7–2.3	–	9.2–26.6
Huang et al. (2015)	378	10.66–33.84	–	16.1–43.14	–
Norton et al. (2014)	370	–	–	10.32–42.41	–
Nawaz et al. (2015)	175	4–47	–	16-55	–
Pradhan et al. (2020)	485	–	1.07–5.38	–	7.43–27.97

leptokurtic and negatively skewed distribution of the trait in a generation mean analysis (GMA) while for Zn content, positive skewness with leptokurtic distribution indicated the involvement of a comparatively fewer number of segregating genes with decreasing effects. They have also suggested the absence of additive epistasis interaction for Zn based on the non-significant deviation of coefficient of skewness from zero. Recently, Kumar et al. (2020) conducted a GMA using the Fe and Zn data recorded on six basic generations of the cross Khusisoi-RISareku/IR91175-27-1-3-1-3 and reported greater magnitude of dominance gene effects over the additive gene effects for both grain Fe and Zn content. Duplicate gene action was suggested for both Fe and Zn with the dominance gene action and dominance × dominance interaction effects acting in opposite directions. The

authors suggested postponing the selection to later generations would be effective in improving Fe and Zn through recombination breeding.

4 Molecular Mapping for Fe and Zn

The advent of DNA markers accelerated the development of molecular linkage maps that aided the mapping of quantitative trait loci (QTLs) for various traits in rice. From the literature survey, when compared to other agronomic traits, it appears that the necessity for the genetic mapping of micronutrient enrichment in rice was realized much later in the year 2008 with the very first study of QTL mapping for grain Fe and Zn content (Lu et al. 2008). Till date, a total of 20 studies have been published on QTL mapping experiments for Fe and Zn, which is significantly low when compared to other traits including yield, biotic and abiotic stress resistance. In these studies, several QTLs governing Fe and Zn either in brown rice or polished rice have been mapped using different bi-parental mapping populations including F_2, Recombinant Inbred Lines (RILs), Backcross Inbred Lines (BILs), Doubled Haploids (DHs) and Introgression Lines (ILs) (Table 3.2). A total of 44 QTLs were reported for Fe content in brown rice across the studies. QTLs were located on all the chromosomes of rice except chromosome 11. While majority of the QTLs reported were located on chromosome 2 (8 QTLs) followed by chromosome 1 (7 QTLs), very few QTLs were reported on chromosomes five (1 QTL) and 9 (2 QTLs). Chromosome 6 and 12 had five QTLs each followed by Chromosome 7 (4 QTLs). Chromosomes 3, 4, 8 and 10 had three QTLs each.

The phenotypic variance explained (PVE) by these QTLs for Fe content in brown rice varied from 2.4% to 71% (Kumar et al. 2014; Anuradha et al. 2012). Of all the QTLs, 12 QTLs had minor effects explaining PVE of less than 10%, 17 QTLs had moderate effects with 10–30% PVE while the remaining seven were major effect QTLs with >30% PVE. The additive effect of the QTLs ranged from 0.04 (Dixit et al. 2019) to 149.97 (Kumar et al. 2014) and 17 of the 44 QTLs have additive effects more than 5%. In contrast to a significantly higher number of QTLs for Fe content in brown rice, only 25 QTLs were reported for Fe content in polished rice; four QTLs were reported on chromosome 1 followed by three QTLs each on chromosomes 3, 4, 6, 7 and 9 while chromosomes 8 and 11 harboured two QTLs each while chromosomes 2 and 12 had one QTL each on them. None of the studies reported QTLs on chromosomes 5 and 10. The PVE explained by these QTLs was in the range of 5.45–26.25% (Wattoo et al. 2019). Of the 25 QTLs, nine were with the minor effect of <10% PVE explained while the remaining 16 QTLs have moderate effect explaining PVE 10–30%. The additive effect of the QTLs explained was in the range of 0.1 (Lee et al. 2020) to 5.29 (Wattoo et al. 2019).

For grain Zn content, a total of 35 and 38 QTLs were reported in brown rice and polished rice respectively. For Zn in brown rice, the highest number of five QTLs were reported on chromosome 3 followed by four QTLs each on chromosomes 1, 6 and 12, three QTLs each on chromosomes 2, 7, 8 and 10, two QTLs on chromosomes 4, 5 and 9 and no QTLs on chromosome 11. The PVE explained by

Table 3.2 Details of QTLs identified for Fe and Zn content through molecular mapping studies

S. no.	Mapping population type	Population size	Parents	QTL	QTL position	R^2	Additive effect	Positive allele	Reference
Fe_Brown rice									
1	BC2F5	111	RP-Bio226 / Sampada	*qFe1.1*	RM562-RM11943	17.1	0.11		Dixit et al. (2019)
				qFe1.2	RM294A-RM12276	14	1.11		
				qFe6.1	RM8226-RM400	6.6	−0.04		
				qFe6.2	RM400-RM162	5.1	0.65		
2	BIL	202	Xieqingzao B / DWR	*qFe3*	RG510-RZ251	28.2	5.41		Hu et al. (2016)
				qFe6	RG123-RG172	16.7	2.01		
				qFe9	RM215-RG451	6.1	−1.59		
3	F2	247	PAU201 / Palman579	*qFe2.1*	RM53-RM521	21.4	135.26	Palman579	Kumar et al. (2014)
				qFe2.2	RM263-RM221	6.9	149.97	Palman579	
				qFe2.3	RM221-RM208	26.8	147.47	Palman579	
				qFe3.1	RM489-RM7	8.8	145.21	Palman579	
				qFe7.1	RM481-RM418	2.4	110.58	Palman579	
				qFe10.1	RM474-RM184	9.2	−143.23	PAU201	
				qFe10.2	RM228-RM496	18.1	149.91	Palman579	
				qFe12.1	RM491-RM519	16.9	3.04	Palman579	
4	DH	120	Chunjiang 06 / TN1	*qFe1.1*	RM246-RM5461	15.7	−1.6312	Chunjiang 06	Du et al. (2013)
				qFe6.1	RM340-RM494	10.6	−1.3498	Chunjiang 06	
				qFe8.1	RM4085-RM1111	22.3	1.9368	TN1	
5	RIL	168	Madhukar / Swarna	*qFe1.1*	RM243-RM488	69	−59.05	S	Anuradha et al. (2012)
				qFe1.2	RM488-RM490	69.2	−59.05	S	
				qFe5.1	RM574-RM122	69.2	−59.1	S	
				qFe7.1	RM234-RM248	69	59.1	M	
				qFe7.2	RM248-RM8007	69	59.1	M	
				qFe12.1	RM17-RM260	71	−59.5	S	
				qFe12.2	RM260-RM7102	71	−59.5	S	

(continued)

Table 3.2 (continued)

S. no.	Mapping population type	Population size	Parents	QTL	QTL position	R^2	Additive effect	Positive allele	Reference
6	ILs	85	Teqing / *Oryza rufipogon*	*qFe2.1*	RM6641	7	1.15	*Oryza rufipogon*	Garcia-Oliveira et al. (2009)
				qFe9.1	RM296	5	−0.78	Teqing	
7	DH	120	Samgang / Nadgong	*Fe6*	6022–6023	13	2.6	Samgang	Qin et al. 2008
8	DH	129	IR64 / Azucena	*qFe1*	RM34-RM237	15	5.901	Azucena	Stangoulis et al. (2007)
				qFe2	RM53-RM300	16.5	1.644		
				qFe8	RM137-RM325A	18.3	2.542	Azucena	
				qFe12.1	RM270-RM17	13.8	1.456	Azucena	
				qFe12.2	RM235-RM17	12.8	5.407	Azucena	
9	ILs	123	TeQing / Lemont	*qFe1*	RM5		−0.29		Zhang et al. (2014)
				qFe2.1	RM452		−0.24		
				qFe2.2	RM6933		−0.32		
				qFe4.1	RM3317		−0.25		
				qFe4.2	RM3217		0.24		
				qFe7	RM248		−0.21		
				qFe8	RM44/RM1148		−0.30		
				qFe10	RM1108		−0.37		
10	ILs	280	Lemont / TeQing	*qFe2.1*	RG437	5.2	−0.31		Zhang et al. (2014)
				qFe3	RG104	3.9	−0.25		
				qFe4	RZ740b	3.3	0.26		
Zn_Brown rice									
1	DH	123	Cultivar 93-11 / Milyang 352	*qZn3.1*	ad03013905-ad03014175	18	−0.10	Milyang 352	Lee et al. (2020)
2	RIL	244	Zhenshan 97B / Miyang 46	*qZn1.1*	RZ460	5.47	0.94		Wang et al. (2020)
				qZn1.2	RM265	8.67	0.63		
				qZn5	RM164	8.07	1.16		

3	BC2F5	111	RP-Bio226 / Sampada	*qZn1.1*	RM294A-RM12276	14.3	3.2		Dixit et al. (2019)
				qZn6.1	RM8226-RM400	34.2	1.21		
				qZn6.2	RM400-RM162	2.9	0.06		
4	BRIL	151	Nipponbare / *O. meridionalis* W1627	*qGZn9*	RM24085-RM566	21.9	4.6	W1627	Ishikawa et al. (2017)
				qGZn10	RM171-RM590	15	3.3	W1627	
				qGZn2.1	RM573	15.2	5.8	W1627	
				qGZn2.2	RM6	17.6	5.1	W1627	
5	BIL	202	Xieqingzao B / DWR	*qZn3*	RG348-RG450	5.5	1.6		Hu et al. (2016)
				qZn4	RM303-RG214	5.3	−2		
				qZn6	RG172-RM340	11.8	2.02		
				qZn12	RM19-RM247	9.2	2.13		
6	F2	247	PAU201 / Palman579	*qZn2.1*	RM521-RM29	5.1	−69.53	PAU201	Kumar et al. (2014)
				qZn10.1	RM474-RM184	19.1	−70.72	PAU201	
				qZn10.2	RM496-RM591	4.7	−69.39	PAU201	
7	DH	120	Chunjiang 06 / TN1	*qZn3.1*	RM3280-RM282	10.8	−8.9171	Chunjiang 06	Du et al. (2013)
				qZn8.1	RM1111-SSIII2	15.1	9.637	TN1	
				qZn9.1	RM278-RM3919b	15.4	9.7761	TN1	
				qZn1.1	RM1349-RM246	14	−4.2321	Chunjiang 06	
				qZn3.2	RM7000-RM514	13.5	−4.1324	Chunjiang 06	
				qZn8.2	RM6356-RM1376	12.5	3.9369	TN1	
8	RIL	168	Madhukar / Swarna	*qZn3.1*	RM7-RM517	31	11.01	M	Anuradha et al. (2012)
				qZn7.1	RM234-RM248	35	13.3	M	
				qZn7.2	RM248-RM8007	35	13.3	M	
				qZn7.3	RM501-OsZip2	29	−11.4	S	
				qZn12.1	RM17-RM260	35	−16.2	S	
				qZn12.2	RM260-RM7102	34	−17.1	S	

(continued)

Table 3.2 (continued)

S. no.	Mapping population type	Population size	Parents	QTL	QTL position	R^2	Additive effect	Positive allele	Reference
9	DH	127	ZYQ8 / JX17	*qZn4*	CT206-G177	10.83	0.35	JX17	Zhang et al. (2011)
				qZn6	RZ516-G30	12.38	0.37	JX17	
10	ILs	85	Teqing / *Oryza rufipogon*	*qZn5.1*	RM1089	5	−2.44	Teqing	Garcia-Oliveira et al. (2009)
				qZn8.1	RM152	11	3.86	*Oryza rufipogon*	
				qZn12.1	RM3331	11	1.29	*Oryza rufipogon*	
Fe_Milled rice									
1	DH	148	IR05F102 / IR69428	*qFe9.1*	9,809,545–9,819,278	11.79	−0.25	IR69428	Calayugan et al. 2020
				qFe12.1	12,702,072–12,732,307	13.34	−0.2	IR69428	
2	DH	123	Cultivar 93-11 / Milyang 352	*qFe3.1*	ad03014175-KJ03_069	10.7	−0.10	Milyang 352	Lee et al. 2020
3	DH	107	Goami 2 / Hwaseonchal	*qGIC9*	9,784,212–11,435,014	11.25	−0.97	Hwaseonchal	Jeong et al. (2020)
				qGIC11	25,441,582–28,485,469	20.54	−1.02	Hwaseonchal	
4	F2	213	Super basmati and IRBB-57	*qFe1*	RM580-RM81	9.65	2.15		Wattoo et al. (2019)
				qFe3	RM211-RM233	26.25	1.69		
				qFe4	RM489-RM545	11.26	0.65		
				qFe6	RM471-RM417	5.45	4.36		
				qFe7	RM109-RM413	7.46	5.29		
				qFe8.1	RM511-RM260	10.45	0.6		
				qFe8.2	RM309-RM463	13.45	2.3		
5	DH	130	PSBRc82 / Joryeongbyeo	*qFe4.1*	4,733,006–4,743,351	9.4	0.4	PSBRc82	Swamy et al. (2018)

6	RIL		Bala / Azucena	*qFe1*	C949	16.2		Azucena	Norton et al. (2010)
				qFe3	R1618	21.4		Bala	
				qFe4	RM349	9.7		Bala	
				qFe7	R1440	15.5		Bala	
7	RIL	241	Zhengshan 97 / Minghui 63	*qFe1*	RG236-C112	25.81	3.36	Minghui 63	Lu et al. (2008)
				qFe9	C472-R2638	11.11	−2.2	Zhenshan 97	
8	BILs	201	IR75862 / Ce258	*qFe1*	RM302-RM486	8	0.706		Xu et al. (2015)
				qFe2	RM154-RM211	7.5	0.271		
				qFe6	RM3-RM340	18.3	0.633		
				qFe7	RM134-RM1132	6.3	0.449		
9	BILs	200	IR75862 / ZGX1	*qFe11*	RM441-RM202	5.5	−0.0.127		Xu et al. (2015)
				qFe6	RM3-RM340	10.2	−0.134		
Zn_Milled rice									
1	DH	107	Goami 2 / Hwaseonchal	*qGZC7*	23,173,313–25,328,698	18.85	−1.59	Hwaseonchal	Jeong et al. (2020)
2	DH	148	IR05F102 / IR69428	*qZn1.1*	id1008679-439764	8.96	−0.79	IR69428	Calayugan et al. (2020)
				qZn5.1	4,904,312–4,908,650	12.15	−0.84	IR69428	
				qZn9.1	9,809,545–9,819,278	13.79	−0.096	IR69428	
				qZn12.1	c12p4887439-12172332	15.26	−0.77	IR69428	
3	F2	213	Super Basmati / IRBB-57	*qZn1*	RM81-RM140	11.12	2.9		Wattoo et al. (2019)
				qZn4.1	RM523-RM489	7.35	3.2		
				qZn4.2	RM338-RM339	6.12	0.9		
				qZn6	RM185-RM471	7.65	0.43		
				qZn7	RM413-RM448	10.24	0.53		
				qZn8	RM179-RM277	9.23	4.06		

(continued)

Table 3.2 (continued)

S. no.	Mapping population type	Population size	Parents	QTL	QTL position	R^2	Additive effect	Positive allele	Reference
4	DH	97	PSBRc82 / IR69428	*qZn11.1*	10858811-id11000778	22.8	−1.6	IR69428	Swamy et al. (2018)
5	DH	130	PSBRc82 / Joryeongbyeo	*qZn2.1*	2110566-id2009463	17.3	−1	Joryeongbyeo	Swamy et al. (2018)
				qZn6.1	6,025,827–6,047,367	15.3	−1.5	Joryeongbyeo	
				qZn12.1	13,048,465 13,057,679	12.2	−0.9	Joryeongbyeo	
6	BIL	202	Xieqingzao B / DWR	*qZn7*	RM11-RM234	7.1	1.86		Hu et al. (2016)
				qZn10	RM1125-RM6704	8.1	2.41		
7	BILs	201	IR75862 / Ce258	*qZn3*	RM293-RM85	14.4	1.061		Xu et al. (2015)
				qZn6	RM3-RM340	24.8	1.743		
				qZn7	RM134-RM1132	2	1.621		
				qZn8	RM407-RM152	18	0.98		
				qZn12	RM1337-RM3409	12.2	0.907		
8	BILs	200	IR75862 / ZGX1	*qZn3*	RM293-RM85	11.1	0.108		Xu et al. (2015)
				qZn7	RM134-RM1132	7	1.036		
				qZn6	RM3-RM340	7.3	−0.562		
				qZn8	RM407-RM152	11.2	3.113		
9	RILs	243	Zhenshan 97 / Milyang 46	*qZn1*	RM315-RZ538	4.8	1.02		Huang et al. (2015)
				qZn3	RZ142-RZ613	11.7	1.59		
				qZn5	RG119-RG346	5	1.05		
				qZn6	RM204-RM225	6.5	1.18		
				qZn11	RM7557-RZ816	4.4	−0.98		

10	RIL		Bala / Azucena	*qZn6*	AB0601	14.7		Bala	Norton et al. (2010)
				qZn7	G20	11.4		Bala	
				qZn10.1	G1082	11.2		Bala	
				qZn10.2	C223	14.8		Bala	
10	RIL	241	Zhengshan 97 / Minghui 63	*qZn5*	R3166-RG360	12.34	−2.37	Zhenshan 97	Lu et al. (2008)
				qZn7	RM234-R1789	5.3	−1.55	Zhenshan 97	
				qZn11	C794-RG118	18.61	2.91	Minghui 63	

these QTLs was in the range of 2.9% (Dixit et al. 2019) to 35% (Anuradha et al. 2012) while the additive effect was ranged from 0.1 (Lee et al. 2020) to 70.72 (Kumar et al. 2014). Of the 35 QTLs, 10 QTLs showed minor effects (PVE <10%), 19 QTLs had moderate effects (PVE 10–30%) and remaining six QTLs showed major effects (>30% PVE). The highest number of QTLs for grain Zn in polished rice was observed on chromosomes 7 (7 QTLs) followed by chromosome 6 (6 QTLs), chromosomes 1, 3, 5, 8, 10, 11 and 12 (3 QTLs), chromosomes 4 (2 QTLs), while chromosomes 2 and 9 (1 QTL). The QTLs were of minor (15 QTLs) and moderate effects (23 QTLs) with PVE ranging from 2% to 24.8% (Xu et al. 2015) while additive effectiveness ranged from 0.09 (Calayugan et al. 2020) to 4.06 (Wattoo et al. 2019).

Few studies also reported QTLs consistent across the seasons and locations in addition to environment-specific QTLs. Garcia-Oliveira et al. (2009) reported 3 consistent QTLs over the seasons, one for Fe on chromosome 2 and 2 for Zn on chromosomes 5 and 8 based on the analysis in brown rice. Hu et al. (2016) reported one QTL on chromosome 10 common for Zn content in both brown and polished rice and stably expressing across locations. Xu et al. (2015) reported common QTLs across the environments for Fe (1 QTL) and Zn (3 QTLs). Swamy et al. (2018) identified one QTL on chromosome two common for two seasons for Zn content. Calayugan et al. (2020) identified two QTLs for grain Zn in polished rice stably expressing across three seasons while Dixit et al. (2019) reported two QTLs on chromosomes 1 and 6 stable across the seasons for brown rice Zn content. Jeong et al. (2020) reported consistent QTLs across the seasons for Fe on chromosomes 9 and 11 and Zn on chromosome 7. The significantly lower proportion of QTLs expressing consistently across locations and seasons indicates the presence of a prominent role of genotype/environment interaction influencing the traits.

5 Genome-Wide Association Studies (GWAS) for Fe and Zn

Rice is the first food crop with its whole genome sequenced (Goff et al. 2002; Yu et al. 2002; International Rice Genome Sequencing Project 2005). The recent advancements in next-generation sequencing (NGS) technologies and single nucleotide polymorphism (SNP) genotyping platforms accelerated the pace of QTL/gene identification (Thomson 2014). GWAS is a powerful mapping technique to unravel the molecular mechanisms underlying complex traits. Through exploiting numerous ancestral recombination events, GWAS has the potential to dissect out genetic mechanism of inheritance of a complex trait and effectively localize it to a narrow genomic region.

Initial GWAS studies have adopted SSR markers to identify QTLs governing Fe and Zn. A USDA rice mini-core collection containing 219 accessions was assessed for Fe (16–55 ppm) and Zn (4–47 ppm) in brown rice and GWAS performed using 155 SSRs could identify 30 and 7 QTLs for Fe and Zn respectively (Nawaz et al. 2015). In another study, a set of 378 brown rice accessions was investigated for Fe (10.66–33.84 ppm) and Zn (16.1–43.14 ppm) using 143 SSR markers to detect

three QTLs for Fe (on chromosome 5, 9 and 12) and four QTLs for Zn (on chromosome 4, 6, 7, 9 and 11) (Huang et al. 2015). Further, a panel of 102 germplasm lines was used for association mapping of grain Fe (1.07–5.38 ppm) and Zn (7.43–27.97 ppm) content in the milled rice using 100 SSR markers, which could identify 10 QTLs for Fe and 7 QTLs for Zn with PVE of 5.5–14.3% and 5.05–10.7% respectively (Pradhan et al. 2020). In recent years, the availability of abundant re-sequencing information has enabled identification of millions of sequence polymorphisms including SNPs across the rice genome. This ample re-sequence data provided the basis for the development of high-throughput genotyping assays suitable for various downstream applications. Several such SNP arrays with varying densities are available in rice including the low and medium density range in GoldenGate 1536 SNPs (Zhao et al. 2010), 384-plex BeadXpress (Chen et al. 2011), C6AIR (Thomson et al. 2017), two Illumina Infinium-based 6K arrays, and the RiceSNP6K (Yu et al. 2014). These have been utilized for QTL mapping, marker-assisted backcrossing (MABC), diversity analysis and also pedigree verification in some cases. The high-density array platforms such as 44K array (GeneChip Rice 44K) (Zhao et al. 2011), Affymetrix 50K arrays (Singh et al. 2015), RiceSNP50K (Chen et al. 2014) and 700 K High-Density Rice Array (HDRA700K) (McCouch et al. 2016), are principally being deployed for GWAS (Crowell et al. 2016). These SNP arrays have been highly informative across diverse rice germplasm covering different rice sub-populations and are being used to dissect phenotype to genotype association, genetic and phylogenetic relationships. For instance, a study identified six QTLs for Fe (2 QTLs on chromosome 6, one QTL each on chromosome 1, 3, 7 and 10) showing favourable allele effect (FAE) ranging from −0.158 to 0.337, and four QTLs for Zn (one QTL each on chromosome 1,7,9 and 12) showing FAE ranging from −0.89 to 1.795 by assessing a set of 698 germplasms accessions comprising two subsets, *indica* (265 accessions from the 3000 Rice genomes project, 2014) and *japonica* (433 accessions), for Fe (0.9–9.1 ppm) and Zn (5.8–29.6 ppm) in a GWAS analysis using 13K SNPs common to both the sets (Zhang et al. 2018a). A recent study assessed a set of 192 Indian rice germplasm for Fe and Zn in both brown rice and milled rice and using a high density genotyping chip consisting of 50K SNP markers (Singh et al. 2015) identified 13 MTAs for Fe (6 in BR and 8 in MR) with PVE ranging from 2.1% to 53.3%, as well as 16 MTAs (11 in BR and 5 in MR) for Zn showing a PVE ranging from 2.3% to 47.6% (Bollinedi et al. 2020b). In another study, GWAS of 152 coloured rice accessions were carried out for Fe (0.7–2.3 ppm) and Zn (9.2–26.6 ppm) using 22,112 SNPs to map two QTLs (on the chromosome for 6 and 12) with PVE ranging from 10.3–10.6% and five QTLs (2 QTLs on chromosome 12; one each on chromosome 1, 6 and 11) with PVE ranging from 11.9–17.9% respectively (Descalsota et al. 2018). In yet another study, approximately 300 accessions were investigated for Zn showing 40% variation at different locations and GWAS was done using 36,900 SNPs, which could identify 198 SNPs associated with Zn content, out of which only 2 SNPs (on chromosome 7 and 90) showed association across the locations (Norton et al. 2014). GWAS has also been carried out in Multi-parent Advanced Generation Intercross (MAGIC) populations that have

the advantage of the relatively wide genetic background without a significant population structure. Descalsota et al. (2018) reported seven QTLs (2 QTLs on chromosome 3 and 9; one QTL each on chromosome 7, 10 and 11) for Fe and seven QTLs (2 QTLs on chromosome 2 and one each on chromosome 1, 2, 4, 7 and 12) for Zn using a set of 144 MAGIC Plus lines genotyped with 14,242 SNPs. Further, a total of 1027 MAGIC RILs genotyped for 66,309 SNP markers were used in association analysis leading to the identification of three QTLs, one each on chromosome 1, 5 and 7 with PVE ranging from 17.5–20.10% (Zaw et al. 2019).

6 Functional Genomics of Grain Fe and Zn

Understanding the molecular mechanisms of mineral uptake by roots, intercellular transport, distribution and loading into grains is essential to design an efficient breeding strategy for biofortification of Fe and Zn. In the earth's crust, Fe stands at fourth position in terms of abundance after oxygen, silicon and aluminium while Zn stands at 24th position. In the soil interface, Fe exits in two different ionic forms, the insoluble ferric ion (Fe^{3+}) and the soluble ferrous ion (Fe^{2+}) (Hori et al. 2015) whereas Zn is predominantly present as a divalent cation (Zn^{2+}). Plants have evolved two different mechanisms of Fe uptake from soil viz. reduction-based strategy I and chelation-based strategy II (Fig. 3.2). The strategy I is more common in non-graminaceous dicotyledonous plants and involves two co-ordinately induced actions for Fe acquisition. Firstly, the sparingly soluble Fe^{3+} is converted to its reduced Fe^{2+} form, independently by two groups of enzymes. In acidic soils, the membrane-bound enzyme Ferric Reductase Oxidase (*FRO*) catalyses the reduction of ferric chelates and release Fe^{2+} ions (Robinson et al. 1999). In soils with alkaline pH, an enzyme H^+ ATPase (AHA) causes acidification of soil rhizosphere by releasing protons and solubilize Fe^{3+} (Santi and Schmidt 2009). The subsequent absorption of solubilized Fe by roots is mediated by the Iron Regulated Transporter1 (*IRT1*) (Connolly and Guerinot 2002; Kobayashi and Nishizawa 2012). Further, the role of phenolic compounds including flavins and phenylpropanoids in the uptake of Fe from immobile Fe sources has been elucidated using homozygous mutants of the gene F6'H1 in the phenylpropanoid pathway and *pleiotropic drug resistance 9* gene encoding a transporter for the pathway products (Ishimaru et al. 2011; Rodríguez-Celma and Schmidt 2013; Rodríguez-Celma et al. 2013). These mutants were unable to excrete phenolics from roots and showed compromised growth on media supplied with insoluble Fe sources (Rodríguez-Celma et al. 2013).

Graminaceous plants including rice adopt the strategy II for Fe acquisition from soils. This involves secretion of Fe_{3+} chelators called phytosiderophores belonging to the mugineic acid (MA) family. Deoxymugineic acid (DMA), a phytosiderophore, is synthesized from S-adenosyl methionine (SAM) through a series of enzymatic reactions (Bashir et al. 2006). Firstly, the nicotianamine (NA) synthesis is mediated by the enzyme NA synthase (NAS) that catalyses the trimerization of SAM obtained

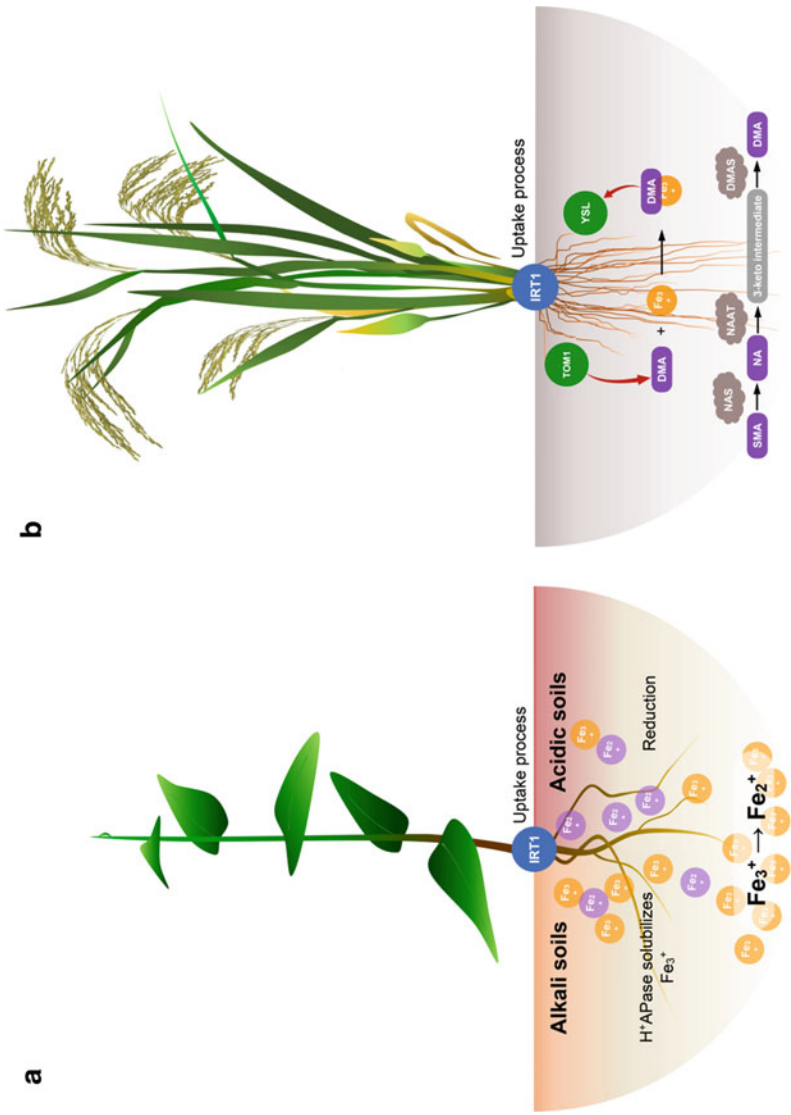

Fig. 3.2 Strategies of Fe uptake by plant roots: reduction-based strategy I (**a**) and chelation-based strategy II (**b**)

from the L-methionine cycle (Takizawa et al. 1996; Higuchi et al. 1999; Takahashi et al. 1999; Inoue et al. 2003, 2008). An amino group is then transferred to NA by the enzyme NA aminotransferase (NAAT) to synthesize a 3″-keto intermediate (Inoue et al. 2008) which is further converted to DMA by the action of the enzyme DMA synthase (DMAS) (Bashir et al. 2006). DMA is the only type of phytosiderophore reported in rice, wheat and maize while in barley and rye DMA is further converted into its hydroxylate derivatives like 3-hydroxymugineic acid and 3-epi-hydroxymugineic acid that impart higher tolerance to Fe chlorosis (Nakanishi et al. 2000; Ueno et al. 2007; Nozoye et al. 2017). Plants secrete phytosiderophores into the root rhizosphere through a *TOM1/OsZIFL4* transporter, belonging to the major facilitator super family (MFS) (Pao et al. 1998; Furrer et al. 2002). Subsequently, the phytosiderophores chelate to Fe^{3+} ions in the rhizosphere forming the soluble 'Fe^{3+}-phytosiderophore' complex, which is readily absorbed by the roots with the help of plasma membrane-localized transporter proteins belonging to the Yellow Stripe 1 (YS1) family called yellow stripe like proteins (YSLs) (Inoue et al. 2009; Lee et al. 2009a; Nozoye et al. 2011). So far, 18 YS1-like (*OsYSL*) genes were identified in rice (Koike et al. 2004). The expression of *OsYSL15* is induced in exodermis and phloem cells under Fe deficiency, suggesting its role in the acquisition of Fe^{3+}-DMA complex and its transport into the phloem (Inoue et al. 2009). *OsYSL16* is highly similar to *OsYSL15* and acquires Fe^{3+}-DMA from the rhizosphere. A member of natural resistance-associated macrophage protein (NRAMP) family proteins, *OsNRAMP* whose expression is restricted to root epidermis, endodermis and cortex contributes to Fe uptake by roots (Ishimaru et al. 2012).

Unlike other grass species, rice adopts a combined strategy for Fe acquisition, owing to its adaptation to flooded environments (Wairich et al. 2019; Zaharieva and Römheld 2000). Waterlogging leads to persistence of anaerobic conditions in the paddy fields under which Fe exists as readily available Fe^{2+} form (Ishimaru et al. 2006; Wang et al. 2020). Despite the presence of functional strategy II system, rice adopts strategy I for the acquisition of abundant Fe^{2+} ions under deoxygenated conditions with the help of trans-membrane *OsIRT1* and *OsIRT2* transporters whose expression has been reported to be induced under Fe deficiency (Bughio et al. 2002; Ishimaru et al. 2006; Liu et al. 2019). Nevertheless, under upland conditions rice takes up Fe^{3+} adopting strategy II.

Plants acquire Zn either as divalent cationic form Zn^{2+} or Zn-DMA complex. Transporters belonging to the ZIP (Zinc-regulated transporters, Iron-regulated transporter-like Protein) family are responsible for Zn uptake from the rhizosphere in rice (Bashir et al. 2012; Humayan Kabir et al. 2014). *OsZIP1* was the first ZIP transporter reported for its role in root uptake by expressing in root exodermis. Its expression is found upregulated under Zn deficiency (Ramesh et al. 2003). Recently, Huang et al. (2020) reported another influx transporter *OsZIP9* involved in Zn uptake under Zn limited conditions of rice soils. Further, the Fe transporter *OsIRT1* also permits Zn uptake as elevated levels of Fe and Zn was observed in the *OsIRT1* overexpressed lines (Lee and An 2009). So far, there is no direct evidence supporting the uptake of Zn as Zn-DMA complex in rice although it was

reported in other graminaceous plants including maize and barley (Von Wiren et al. 1996; Suzuki et al. 2006).

After uptake by roots, internal transport of Fe and Zn occurs through the vascular bundles including xylem and phloem and ultimately distributed to the leaves and seeds. The radial intercellular transport occurs predominantly through symplast while movement through apoplast is impeded by the presence of Casparian strips in the root cortex (Enstone et al. 2002). Internal mobilization of root acquired Fe is mediated by various chelators like citrate, NA, DMA and phenolic acids. A plasma membrane-localized citrate transporter FRD3-LIKE protein 1 (*OsFRDL1*) transports citrate from root pericycle into xylem where it chelates Fe and thereby facilitates efficient root to shoot translocation by impeding Fe precipitation in the xylem sap (Yokosho et al. 2009, 2016). In addition to its role as an intermediate in PS biosynthesis, NA also assists in the intercellular transport of Fe through Fe-NA complexes. The efflux of NA into xylem is mediated by OsENA1 (Nozoye et al. 2011), although Fe bound to NA is not detected in the xylem (Ariga et al. 2014). A portion of the Fe from xylem is loaded into phloem for distribution to leaves and reproductive organs including seeds. Therefore, Fe pools in the seeds are contributed by both xylem and phloem transport. While the concentration of Fe in xylem directly influences the Fe concentration of both shoots and seeds, phloem feeding to seed Fe pools is determined through two routes viz., the shoot Fe accumulation and remobilization from senescing older leaves (Grillet et al. 2014). It has been reported that the YSL family of transporters not only facilitate root Fe uptake as Fe^{3+}-DMA complexes but also are essential for unloading of the Fe into the phloem. *OsYSL2*, a member of the YSL family, is expressed in phloem cells and developing seeds and promotes the transport Fe^{2+}-NA and but not Fe^{3+}-DMA (Koike et al. 2004). Subsequently, the Fe^{3+}-DMA complex transporter activity of *OsYSL18* was demonstrated through electrophysiological measurements in the oocytes of *Xenopus laevis*. The significantly higher expression of *OsYSL18* in the flowers rather than shoots implicated its role in Fe translocation in reproductive organs (Aoyama et al. 2009). A portion of the shoot Fe is sequestered into the vacuoles by the vacuolor iron transporter (VIT) proteins, *OsVIT1* and *OsVIT2* (Zhang et al. 2012) and vacuolar mugineic acid transporter, *OsVMT* (Che et al. 2019). In addition to its role in root uptake, higher expression of *OsYSL16* in the vascular bundles implicated its importance in the internal transport of Fe as Fe^{3+}-DMA complex (Lee et al. 2012a). Unlike *OsYSL2*, *OsYSL9* transports Fe as both Fe^{2+}-NA and Fe^{3+}-DMA complexes. The expression of *OsYSL9* was noticed in the scutellar tissue of the embryo as well as in the outer layer of the endosperm surrounding the embryo. Confirming this, the *OsYSL9* knockout mutants showed reduced Fe concentration in the embryo with a concomitant increase in Fe concentration in the residual parts of the brown rice and polished rice. These evidences suggested that *OsYSL9* is indispensable in the internal transport of Fe from endosperm to embryo (Senoura et al. 2017). A recent study reported Fe distribution from older leaves to younger ones is mediated by *OsYSL13* transporter (Zhang et al. 2018b).

7 Zinc Uptake and Translocation

A portion of the Zn acquired by the roots is sequestered into the root vacuoles by a transporter protein belonging to the Heavy Metal ATPase family called *OsHMA3* (Fig. 3.3). This protein is localized in the tonoplast (Ueno et al. 2010) while the remaining Zn is loaded into the xylem by another transporter of the same family, *OsHMA2*, localized in the plasma membrane of root pericycle (Yamaji et al. 2013; Takahashi et al. 2012; Satoh-Nagasawa et al. 2012). Knockdown mutants of *OsHMA2* showed decreased efficiency of root-to-shoot Zn transfer demonstrating the role of *OsHMA2* in loading Zn into the vascular bundles (Takahashi et al. 2012; Satoh-Nagasawa et al. 2012). Another plasma membrane-localized Zn efflux transporter, OsHMA9, shows stele-specific expression and is believed to play a role in loading Zn onto the xylem (Lee et al. 2007). The induction of expression of *OsZIP4* under Zn deficiency conditions demonstrates its role in phloem Zn loading (Ishimaru et al. 2005). The primary source for Zn accumulation in rice grain is still debated as contradictory results were reported by different authors. Some studies claim that the

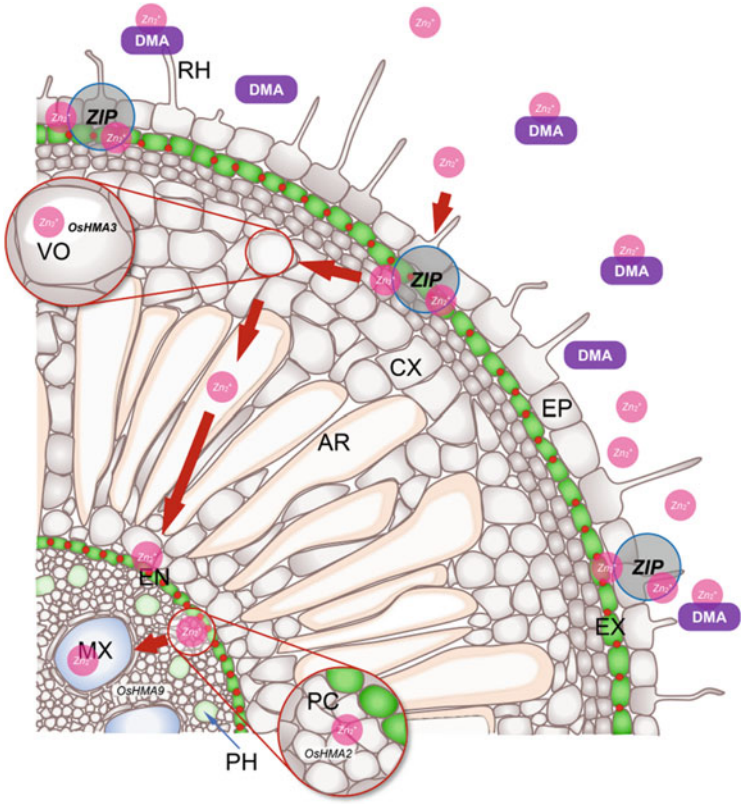

Fig. 3.3 Mechanism of translocation of Zn in rice

main source under Zn sufficient conditions is the root acquired Zn that is transported continuously through the xylem stream (Jiang et al. 2007; Stomph et al. 2009). Conversely, Wu et al. (2010) reported that remobilization from senescing older leaves acts as the main source of Zn in a high grain Zn rice genotype.

8 Genetic Engineering for Fe, Zn and Pro-vitamin A Enhancement

Conventional and molecular breeding attempts so far indicate marginal success for Fe, Zn and pro-vitamin A biofortification in rice. The limited variability for these traits in the rice germplasm, particularly in the endosperm content and their complex polygenic nature of inheritance are implicated as the major reasons (Naqvi et al. 2009). Presence of significant genotype × environment interactions further complicates the process and results in trivial success through conventional approaches. Added to this is the negative association of micronutrient concentration with grain yield, which poses a major challenge to break the undesirable linkage through conventional approaches. Under these circumstances, genetic engineering has been recognized as a viable technology for improvement. A comprehensive description of the attempts done so far is presented below (Table 3.3).

8.1 Grain Fe Content

The very first effort to enhance the endosperm Fe content in rice was carried out by involving the deployment of a soybean *ferritin* gene *SoyferH1,* encoding a Fe storage ferritin protein. Overexpression of *SoyferH1* could sequester almost 4500 atoms of Fe (Goto et al. 1999). Seed-specific expression of *SoyferH1* under the control of an endosperm-specific Glutelin-B1 promoter in a *japonica* cultivar Kitaake resulted in the production of transgenic plants having threefold increase of brown rice Fe concentration over the wild type (Goto et al. 1999). In another study, a 3.7-fold increase in the polished rice could be achieved in the genetic background of *indica* cv. IR68144 (Vasconcelos et al. 2003). Subsequent search of additional ferritin genes identified that *Phaseolus vulgaris ferritin* gene is less efficient to soybean *ferritin* gene with a twofold increase in the Fe content in transgenic *japonica* cultivar Taipei 309 (Lucca et al. 2002). Overexpression of a rice *ferritin* gene *Osfer2* under the control of an endosperm specific *GlutelinA2* (*OsGluA2*) promoter showed 2.09-fold Fe increase in the polished rice grains of transgenic aromatic *indica* cultivar Pusa Sugandhi II (Paul et al. 2012). However, with an intension of enhancing Fe concentration multiple folds, expression of *SoyferH1* under the control of a dual promoter system having *OsGlb1* and *OsGluB1* was attempted, but did not result in significant advantage over single promoter regulated transgenic plants (Qu et al. 2005). Moreover, transgenic plants overexpressing the ferritin gene in the

Table 3.3 Transgenic approaches for enhancing Fe, Zn and provitamin A

Gene	Promoter	Growth condition	Rice cultivar	Brown rice	Polished rice	Method of transformation	Reference
Fe							
Single gene approaches							
GmFERRITIN	Glutelin-B1	Green house	*Japonica* cv. Kitaake	3-fold	2-fold	Agrobacterium-mediated transformation	Goto et al. (1999)
GmFERRITIN	Glutelin-B1	Green house	*Indica* cv. IR68144	2.2-fold	3.7-fold	Biolistic method	Vasconcelos et al. (2003)
PvFERRITIN	Glutelin Gtl	Green house	*Japonica* cv. Taipei 309	2-fold		Agrobacterium-mediated transformation	Lucca et al. (2002)
OsFERRITIN 2	GlutelinA2	Green house	*Indica* cv. Pusa-Sugandhi II		2.09 fold	Biolistic transformation	Paul et al. (2012)
SoyferH-1	GluB-1; Glb-1	Green house	*Japonica* cv. Kitaake	1.5		Agrobacterium-mediated transformation	Qu et al. (2005)
IDS3	CaMV35S	Field conditions (andosol soil)	*Japonica* cv. Tsukinohikari	1.25 fold	1.4 fold	Agrobacterium-mediated transformation	Masuda et al. (2008)
OsNAS2	Maize ubiquitin promoter	Greenhouse	*Japonica* cv. Kitaake		3.0-fold	Agrobacterium-mediated cocultivation	Lee et al. (2012b)
OsNAS3	CaMV35S	Field condition	*Japonica* cv. Dongjin	2.9 fold	2.6 fold	Agrobacterium-mediated transformation	Lee et al. (2009b)
OsYSL2	OsSUT1	Hydropoinc culture	*Japonica* cv. Tsukinohikari		4.4 fold increase	Agrobacterium-mediated transformation	Ishimaru et al. (2010)

Multiple gene approaches							
PvFERRITIN AtNAS1AfPHYTASE	CaMV35S Globulin Globulin	Green house	*Japonica* cv. Taipei 309	2-fold	6.3-fold	Agrobacterium-mediated transformation	Wirth et al. (2009)
GmFERRITIN H2, HvNAS1 OsYSL2	OsGlb1 OsActin1 OsGlb1	Green house	*Japonica* cv. Tsukinohikari		4.4 fold	Agrobacterium-mediated transformation	Masuda et al. (2012)
Zn							
Single gene approaches							
OsNAS3	CaMV35S	Field condition	*Japonica* cv. Dongjin	2.2 fold	2.2 fold	Agrobacterium-mediated cocultivation	Lee et al. (2009b)
OsNAS2	Maize ubiquitin promoter	Greenhouse	*Japonica* cv. Kitaake		–	Agrobacterium-mediated cocultivation	Lee et al. (2012b)
IDS3	CaMV35S	Field condition (andosol soil)	*Japonica* cv. Tsukinohikari	1.3 fold	1.35 fold	Agrobacterium-mediated transformation	Masuda et al. (2008)
OsNAS1	Glutelin B1	Field conditions	*Japonica* cv. Xiushui 110		33–55% higher	Agrobacterium-mediated transformation	Zheng et al. (2010)
OsNAS2	CaMV 35S promoter	Glasshouse	*Japonica* cv. Nipponbare		2-fold	Agrobacterium-mediated transformation	Johnson et al. (2011)
GmFERRITIN H2	Glutelin B1	Glasshouse	*Tropical Japonica* cv. Paw San Yin (Myanmar High Quality Rice)		1.3-fold	Agrobacterium-mediated transformation	Aung et al. (2013)
Multiple gene approaches							
AtIRT1 PvFerritin AtNAS1	MsENOD12B Globulin CaMV35S	Greenhouse	*Japonica* cv. Nipponbare		1.8-fold	Agrobacterium-mediated transformation	Boonyaves et al. (2017)

(continued)

Table 3.3 (continued)

Gene	Promoter	Growth condition	Rice cultivar	Brown rice	Polished rice	Method of transformation	Reference
AtNAS1 *PvFERRITIN CRTI* *ZmPSY*	CaMV 35S OsGLOBULIN OsGLUTELIN	Greenhouse	*Japonica* cv. Nipponbare		1.2 fold	Agrobacterium-mediated transformation	Singh et al. (2017)
OsHMA2	OsSUT1	Field condition	*Japonica* cv. Nipponbare and Sasanishiki		20% increase	Agrobacterium-mediated transformation	Takahashi et al. (2012)
GmFERRITIN H2 *HvNAS1 OsYSL2*	OsGlb1 OsActin1 OsGlb1	Greenhouse	*Japonica* cv. Tsukinohikari		1.6 fold	Agrobacterium-mediated transformation	Masuda et al. (2012)
PvFERRITIN *AtNAS1* *AfPHYTASE*	CaMV35S Globulin Globulin	Greenhouse	*Japonica* cv. Taipei 309	1.6-fold	1.5-fold	Agrobacterium-mediated transformation	Wirth et al. (2009)
Provitamin A							
ZmPsy and *CRTI*	OsGlutelin1	Glasshouse	*Japonica* cv. Kaybonnet		37 µg/g 23-fold	Agrobacterium-mediated transformation	Paine et al. (2005)
AtNAS1 *PvFERRITIN CRTI* *ZmPSY*	CaMV 35S OsGlobulin OsGlutelin1	Greenhouse	*Japonica* cv. Nipponbare		3.4-fold	Agrobacterium-mediated transformation	Singh et al. (2017)
CRTI and *ZmPsy*		Greenhouse	*Japonica* cv. Nipponbare			Agrobacterium-mediated transformation	Singh et al. (2017)
SSU-crtI and *ZmPsy*	OsGlutelin1	Green house	*Japonica* cv. Kitaake		7.9 µg/g		Dong et al. (2020)

endosperm began to show Fe deficiency symptoms in leaves (Qu et al. 2005; Masuda et al. 2013). These results implied that overexpression of *ferritin* gene alone was not sufficient enough to drive enhanced endosperm Fe concentration in rice. It was further understood that poor Fe translocation to endosperm could be a major bottleneck for rice biofortification (Masuda et al. 2008). Recruitment of NAS genes controlling the uptake and long-distance transport of Fe to aerial parts have been attempted to prove this proposition. Transgenic rice plants expressing *NAS1* gene from *Hordeum vulgare* under the control of constitutive CaMV35S promoter showed 5–10-fold increase in the endogenous NA levels in the shoots and seeds with a consequent increase of endosperm Fe content to an extent of three folds over the wild type plants (Masuda et al. 2009). In a separate transgenic approach, transformation of rice with barley *iron deficiency specific clone 3* (*IDS3*) gene involved in mugineic acid biosynthesis resulted in 1.4-fold increase in Fe concentration in polished rice *vis-à-vis* non-transgenic control plants grown under Fe sufficient conditions (Masuda et al. 2008). Lee et al. (2009b) reported that activation tagged lines with enhanced expression of *OsNAS3* showed elevated levels of NA (9.6 folds), Fe (2.9 folds) and Zn (2.6 folds), while activation tagged lines of *OsNAS2* showed threefold increase in Fe content (Lee et al. 2012b). Johnson et al. (2011) evaluated the comparative efficiency of *OsNAS1*, *OsNAS2* and *OsNAS3* genes by using transgenic plants overexpressing individual genes and reported that *OsNAS2* overexpressed lines showed Fe concentration as high as 14–19 μg/g, a 4.2-fold increase over the baseline concentration of 4.5 μg/g. This was the highest ever increase in endosperm Fe concentration achieved through the single gene-based transgenic approach. In yet another study, overexpression of Fe^{2+}-NA transporter gene, *OsYSL2* under the control of a sucrose transporter (*OsSUT1*) promoter, could establish a 4.4-fold increase in Fe concentration in the polished rice (Ishimaru et al. 2010). In addition to these single gene-based approaches, multi-gene-based transgenic lines involving a combination of storage, uptake and transporter genes have shown greater promise in enhancing the endosperm Fe concentration. Wirth et al. (2009) transformed the *japonica* cultivar Taipei 309 with a cassette of three genes viz. *AtNAS1* from *Arabidopsis* (N) under the control of CaMV35S promoter and *PvFERRITIN* from *Phaseolus vulgaris* (F) and *AfPHYTASE* from *Aspergillus fumigates* (P) under the control of globulin promoter. The resultant transgenic lines known as NFP lines showed up to six-fold higher Fe concentrations under hydroponic conditions. Transformation with *NAS*, *OsYSL2* and *ferritin* genes showed a synergistic effect by enhancing the Fe translocation through overproduction of chelators NA and DMA, promoting the Fe flux into endosperm and enhancing its storage in endosperm thereby resulting in a 4.4-fold high Fe concentration in the transgenic lines under field conditions (Masuda et al. 2012).

8.2 Grain Zn Content

The divalent cationic nature of Zn closely resembles that of Fe_{2+} and both the metal ions utilize similar mechanisms for their uptake, translocation and homeostasis. This

is apparent from the simultaneous increase in the Zn concentration of the transgenic lines developed to increase Fe concentration. Overexpression of *FERRITIN* and genes governing Fe uptake and translocation like *OsIRT*, *OsNAS1*, *OsNAS2*, *OsNAS3* and *HvNAS1* showed a concordant increase in Zn concentration (Higuchi et al. 2001; Lee et al. 2009b; Masuda et al. 2009, 2012; Wirth et al. 2009; Zheng et al. 2010; Johnson et al. 2011; Aung et al. 2013; Boonyaves et al. 2017; Singh et al. 2017). Takahashi et al. (2012) demonstrated that overexpression of the rice *Heavy Metal Atpase 2* (*OsHMA2*), a Zn transporter gene regulated under the sucrose promoter *OsSUT1*, was successful in increasing the Zn concentration by 20% in brown rice of transgenic plants over their wild type conuterparts.

8.3 Pro-vitamin A Content

None of the genotypes in the rice gene pool is capable of synthesizing β-carotene in the endosperm despite the presence of the functional genes and pathway in the vegetative tissues. The reason was missing links in the functional carotenoid biosynthesis pathway. Detailed characterization of the pathway intermediates indicated that rice endosperm lacks Phytoene Synthase (PSY), the enzyme that catalyses the condensation of two molecules of Geranyl Geranyl PyroPhosphate (GGPP) into 15-*cis*-phytoene, a colourless carotenoid. In the endosperm tissue, the *OsPsy* gene is transcriptionally repressed leading to its non-expression and therefore the lack of PSY. This has led to a transgenic approach, for restoring the defunct pathway in the rice endosperm. The result was the Golden Rice®, developed by the transformation of a *japonica* cultivar, Taipei 309 with the *PSY* gene (*NpPSY*) from daffodil (*Narcissus pseudonarcissus*) complemented with the *CrtI* gene encoding carotene desaturase sourced from the bacteria *Pantoea ananatis* (formerly *Erwinia uredovora*). In the prototype transgenic lines, the accumulation of β-carotene could be observed in the endosperm, but in limited amounts. Subsequently, it was identified that the *NpPSY*-catalysed biochemical reaction was the major rate-limiting step in β-carotene biosynthesis in the prototype lines. Replacement of the *NpPSY* with *ZmPSY*, the *PSY* gene from maize, lead to a significantly higher accumulation of carotenoids to an extent of 37 μg/g of endosperm. This was demonstrated in the second-generation Golden Rice® lines (GR2) developed in the background of an American long-grain rice variety Kaybonnet (Paine et al. 2005). Afterwards, the Humanitarian Board (HumBo) on Golden Rice® released six transgenic events (G1, R1, L1, T1, W1 and E1) of GR2 for utilization in public sector breeding programs, of which GR2-R1 event was considered as an event for de-regulation (Bollineni et al. 2014). Bollinedi et al. (2017) carried out a comprehensive characterization of backcross derived GR2 lines in the background of a mega rice variety, Swarna and demonstrated that transgene homozygous lines showed inferior agronomic performance with reduced plant height, incomplete panicle exertion, reduced panicle size, increased chaffy grains and greatly reduced single plant yield. However, transgene hemizygotes and the null siblings lacking the transgene had normal phenotypes as that of Swarna. The transgene homozygotes showed altered hormonal homeostasis

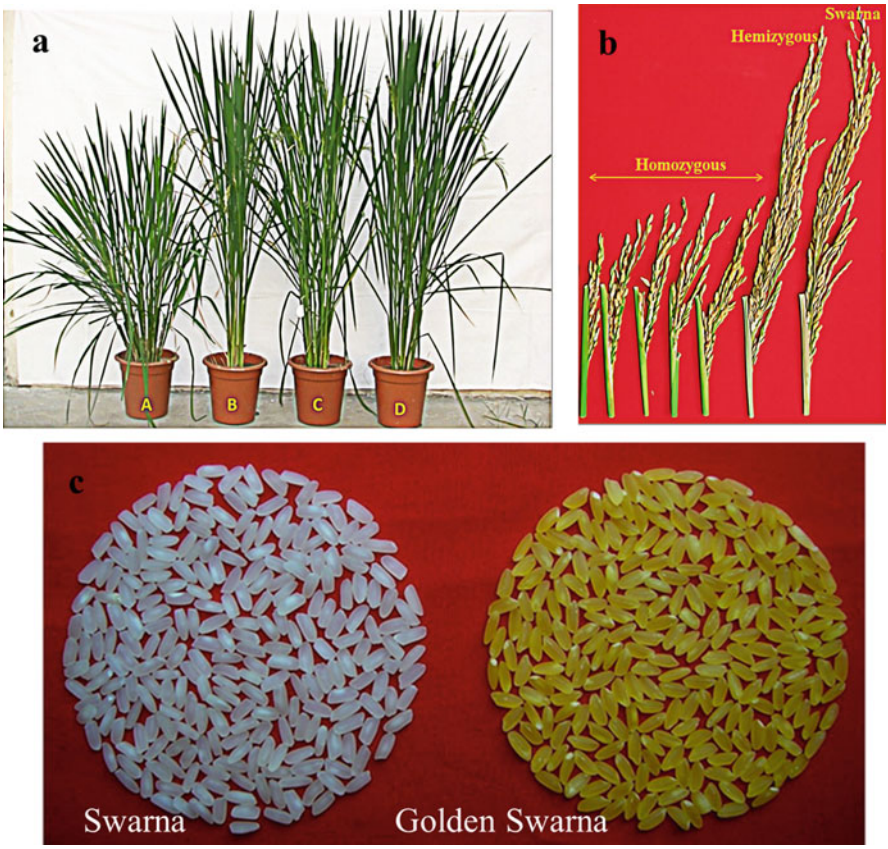

Fig. 3.4 Comparison of plant phenotype among transgene homozygous (A), hemizygous (B), and null (C) lines in relation to the recurrent parent Swarna (D). (**a**) Reduced plant height of transgene homozygous plant in comparison to a hemizygous plant and Swarna. (**b**) Reduced panicle size and poor panicle exertion in a transgene homozygous plant in comparison to a hemizygous plant and the RP Swarna (Fig. 3.4a and 3.4b is reproduced from the article Bollinedi et al. (2017) published in PlosOne. (**c**) Expression of β-carotene in the grains of transgenic Swarna

with reduced level of gibberellins (GA) and increased level of abscisic acid (ABA) concentrations. Consequent molecular analyses revealed that the transgene insertion had disrupted the reading frame of the native *OsAux1* gene that encodes an auxin influx carrier protein (AUX1). Therefore, the transgene homozygotes had produced a non-functional AUX1 protein leading to poor agronomic expression (Fig. 3.4). Despite being controlled by endosperm specific glutelin1 promoter, leaky expression of transgenes occurred in the vegetative tissues leading to a competition for the substrate GGPP between the endogenous *PSY* and transgenic *PSY* in the vegetative tissues. This has led to the disturbance in the homeostasis of various growth regulators including auxins, gibberellins, abscisic acid and cytokinins ultimately affecting the growth and development. Subsequently, transgenic lines expressing provitamin A carotenoids were developed independently by different research

groups globally. In a study by Singh et al. (2017), the *japonica* cultivar Nipponbare was transformed with four genes viz. *AtNAS1* and *PvFERRITIN* governing grain Fe and Zn content along with bacterial *CRTI* and *ZmPSY* involved in carotenoid biosynthesis. The resultant transgenic lines demonstrated a significant increase in the concentration of β-carotene, Fe and Zn in the polished grains. In another recent study, Dong et al. (2020) adopted a *CRISPR-Cas9*-based genome-editing approach in the cultivar, Kitaake, to guide a targeted insertion of a 5.2 kb cassette harbouring the coding sequence of two major genes involved in carotenoid biosynthesis viz., *SSU-crtI* and *ZmPsy* both under the control of the endosperm specific glutelin promoter. The transgenic lines demonstrated accumulation of carotenoids without any off-target effects and yield penalty.

9 Enhancing Nutrient Bioavailability and Quality

The ultimate purpose of biofortification programmes is to meet the minimum dietary requirements of micronutrients to humans, conveniently and constantly. Hence, the actual yardstick for nutrient enrichment in the breeding programmes should be based on the bioavailability of micronutrients rather than the per se quantity. Anti-nutrient factors act as the inhibitors of micronutrient absorption in the human gut. Phytic acid forms one of the major anti-nutrient factors by effectively binding to mineral ions such as Fe, Zn, Ca, Mn, Mg and K and forming mixed salts, leading to impeded absorption of cationic nutrient elements (Ali et al. 2010). Non-ruminants including humans are incapable of digesting the mineral phytate salts due to the lack of digestive enzyme phytase (Mroz et al. 1994; Marounek et al. 2010). Therefore, in humans, ingested phytate salts remain undigested in the intestine and get passed to the lumen and are further excreted, leading to the micronutrient deficiencies. Phytates in the diets of pregnant women significantly impact the Fe, Zn and Ca bioavailability (Al Hasan et al. 2016) leading to ultimate impairment on foetal development and childbirth. Traditional methods like milling, soaking and cooking, fermentation, roasting and germination, etc. have been partially successful in reducing the phytic acid content in food crops (Ogbonna et al. 2012; Kruger et al. 2014; Gupta et al. 2015; Ertop and Bektaş 2018; Nkhata et al. 2018). Nonetheless, the availability of low phytate mutants (LPA) in various crops opened up new avenues for enhancing the bioavailability in staple crops through reducing the phytic acid content (Rasmussen and Hatzack 1998; Raboy 2003; Larson et al. 2000; Pilu et al. 2003; Shi et al. 2003). Induced LPA mutants showing as much as 34–75% reduction in phytic acid content have been identified and characterized in rice (Kim et al. 2008). So far, mutants were identified for the genes *myo*-inositol kinase (*XS-lpa*), 2-phosphoglycerate kinase (two allelic mutants KBNT-*lpa* and XQZ-*lpa*), multi-drug-resistant protein 5 (Z9B-*lpa* and MH-*lpa*) and inositol (1,3,4)P_3 5/6-kinase (*ITPK*) gene involved in phytic acid metabolism (Kim et al. 2008; Zhao et al. 2008; Xu et al. 2009; Kim and Tai 2014). Seed-specific suppression of *RINO1* gene driven by oleosin 18 promoters demonstrated a significant reduction in phytic acid content to an extent of 68% without affecting other parameters like seed weight,

germination, growth and development (Kuwano et al. 2009). However, RNAi directed silencing of *myo*-inositol-3-phosphate synthase (*MIPS*) gene that catalyses the primary step of phytic acid biosynthesis exhibited a remarkable reduction in *myo*-inositol, a key metabolite in signalling pathways including ascorbic acid biosynthesis. In addition to a significant decrease in the seed phytic acid content, the transgenic lines also showed a reduction in the metabolites downstream the *myo*-inositol with subsequent impact on key biological processes of the plant (Ali et al. 2013). Nonetheless, seed-specific silencing of the inositol 1,3,4,5,6-pentakisphosphate 2-kinase (*IP5K*) gene depicted a significant reduction in phytic acid content without any observable negative effects on plant growth and development (Ali et al. 2013). Recently, Jiang et al. (2019) developed CRISPR-Cas9-based mutations targeted to exon 1 of inositol 1,3,4-trisphosphate 5/6-kinase1 gene and observed a significant reduction in the phytic acid content in the gene edited lines. However, the mutants showed significant impairment in plant growth, development and reproduction.

In addition to phytic acid, cadmium (Cd), a toxic element, also acts as an inhibitor of mineral absorption particularly in the absorption of Zn. The major source of Cd intake by human beings is the consumption of foods grown in contaminated soils (Egan et al. 2007), or irrigated with Cd rich brackish/effluent mixed water (Andresen and Küpper 2013). The divalent and cationic nature of Cd resembles the nutrient ions, Fe_{2+} and Zn_{2+}, and competes for the transporters such as IRT, ZIP HMA and NARMP and gets taken up, translocated and accumulated in the rice grain (Uraguchi and Fujiwara 2013; Nakanishi et al. 2006; Ishikawa et al. 2012; Sasaki et al. 2012; Ueno et al. 2010; Miyadate et al. 2011). A different route for minimizing the Cd accumulation in the rice grains has been demonstrated by Uraguchi et al. (2011) through the identification of a low-affinity cation transporter (*OsLCT1*), a plasma-membrane localized efflux transporter. Suppressing the expression of *OsLCT1* ensured a significant reduction in Cd accumulation in rice grain through reducing its transport through the phloem. Interestingly, the transgenic lines did not record any reduction in the content of other metal ions indicating the specificity of *OsLCT1* to Cd transport (Uraguchi et al. 2011, 2014).

With the growing consciousness and awareness on the diet-related non-communicable diseases and disorders, the popularity of health-promoting natural compounds in staple foods is increasing lately. The amount and quality of protein in the diet has a positive impact on the Zn absorption, similar to amino acids such as methionine and histidine, and organic acids such as citric acid. Whereas, polyphenols, a kind of antioxidant compounds can act as inhibitors of Fe absorption (Lonnerdal 2000). On the other hand, dietary fat is essential for the absorption of β-carotene in the intestine, because fat facilitates the incorporation of β-carotene into micelles consisting of free fatty acids, phospholipids, monoglycerides and bile acids (Haskell 2012). The proportion and saturation of fatty acids determine the amount of β-carotene in the miscelle (Yeum and Russell 2002). Further, it is also highly desirable to address the storage losses of β-carotene in Golden Rice®. After evaluating different storage conditions, it was observed that vacuum packing

significantly enhances the retention of β-carotene even after 6 months of storage (Bollinedi et al. 2019).

10 Future Perspectives

In view of the increased global concern of hidden hunger, it is highly desirable to enhance the micronutrient status of the staples that directly target the nutrient security of the economically poor and low-income sections of the society. Biofortification of staple crops has several advantages over other existing strategies like supplementation, industrial fortification and dietary diversification. This improves the reachability and accessibility to the poor, minimal recurring costs and uninfluenced by policy decisions. Nonetheless, the complex genetic nature of the traits poses a major challenge in uplifting the micronutrient concentration above the baseline and achieving the set targets. Committed and concerted efforts are in progress globally to understand the genetics and inheritance pattern of the micronutrient traits. Although significant progress has been made in elucidating the root uptake, translocation and homeostasis of minerals in rice, the mechanisms of grain loading and translocation to different parts of the grain still remains elusive. Bulk of the Fe and Zn in rice is seen compartmentalized in embryo and outer aleurone layers. The genes and transporters that facilitate endosperm translocation in lieu of bran are yet to be deciphered. The anti-nutrients factors like phytic acid that reduce the bioavailability of cationic minerals add another dimension to the challenges of biofortification. The interactions and interlinks among the mineral homeostasis and other nutrients like proteins, lipids and carbohydrates have not been worked out. The crosstalk between β-carotene and vitamin E biosynthesis in rice is to be unfolded owing to their common precursor, GGPP. Recent advancements in various omics technologies would facilitate gaining deeper insights into the aspects of micronutrient accumulation in rice grains that could aid in designing accelerated biofortification programmes for the future.

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Advances in Wheat Biofortification and Mainstreaming Grain Zinc in CIMMYT Wheat Breeding

4

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Abstract

The current and future trends in population growth and consumption continue to increase the demand for wheat, a key cereal for global food security. Wheat products are an important source of essential macro- and micro-nutrients in the human diet. About 2 billion people are deficient in some essential micronutrients including zinc (Zn) and iron (Fe); the magnitude is particularly severe among children, pregnant, and lactating women. Wheat is the second largest produced cereal in India with over 107 million tons during 2019–20 season. It is a primary food staple consumed in India, although consumption varies widely by State. Therefore, biofortified wheat is potentially an ideal vehicle for delivering increased quantities of Zn to young children and their mothers in those States where wheat is a primary staple. The conventional breeding strategies have been successful in the introduction of novel alleles for grain Zn that led to the release of competitive Zn-enriched wheat varieties in South Asia. The major challenge over the next few decades will be to maintain the rates of genetic gains for grain yield along with increased grain Zn concentration to meet the food and nutritional security challenges. Therefore, to remain competitive, the performance of Zn-enhanced lines/varieties must be equal or superior to that of current non-biofortified elite lines/varieties. Since both yield and Zn content are invisible and quantitatively inherited traits except few intermediate effect QTL regions are

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identified for grain Zn, increased breeding efforts and new approaches are required to combine them at high frequency in CIMMYT's elite germplasm, ensuring that Zn levels are steadily increased to the required levels across the CIMMYT breeding pipeline. The addition of Zn as a core trait will require a significant acceleration in the breeding cycle, expanding population sizes, extensive phenotyping for Zn, yield testing, phenotyping for biotic and abiotic stresses, genotyping, molecular-assisted selection, and genomic selection. While continuing to increase agronomic performance, high Zn alleles will be added as a core trait and the Zn content will be increased in breeding lines annually as biofortified varieties with high frequency of elite lines with high Zn high yield potential to be released by partners.

Keywords

Wheat · Genetic diversity · Yield gain · Genomic selection · Nutritional quality

1 Introduction

Micronutrient deficiency or "hidden hunger" affects more than 2 billion people globally and is particularly prevalent in the poorest rural communities of developing countries, where people do not have access to and/or cannot afford a more nutritious diversified diet. Grain zinc (Zn) and iron (Fe) are essential micronutrients, which supplied through wheat can reduce the urgent issue of micronutrient deficiency for about 2 billion people (WHO 2018). The magnitude of Fe and Zn deficiency is particularly severe among children and pregnant and lactating women (Mayer et al. 2008). Biofortified wheat with increased grain Zn and Fe has several potential advantages as a delivery vehicle of Zn and partially for Fe through wheat in South Asia and Ethiopia, and the Zn-enriched wheat can provide up to 50% of daily recommended allowance for humans (Sazawal et al. 2018). Most of the wheat produced in the targeted regions is milled locally, and the use of whole grain wheat flour in food products allows retaining most of the zinc in the grain as these minerals are concentrated in the outer layer of the grain. The consumers in South Asia and Ethiopia prefer flatbreads, such as *chapatti*, *roti*, *nan*, *injera* and other wholegrain products including porridge.

Wheat (*Triticum aestivum* L.) is the world's most important crop species, grown on an area of over 225 million ha and now yielding almost 740 million tons annually (FAOSTAT 2016). Importantly, there has been a steady and highly significant increase in wheat yields, largely due to the release of new improved varieties. Since the early 1960s, there has been little increase in the area sown to wheat, but over the same period, yields have increased almost three-fold (Crespo-Herrera et al. 2017; Sharma et al. 2012). While much of this increase has been through improved agricultural practice, the breeding of new varieties has been crucial. The major challenge over the next few decades will be to maintain these rates of improvement, and the application of the remarkable advances made in molecular genetics and

biotechnology over the last decade to wheat improvement is clearly a key strategy in achieving this.

In recent years, changes in population trends, eating habits, and economic and socio-economic conditions, especially in Africa and Asia have resulted in an increased demand for nutritious healthy diets. Therefore, biofortified wheat with enhanced Zn and Fe concentration could supply essential micronutrients such as Zn, Fe, Mn, Mg, Ca, and vitamin B and E (Bouis and Saltzman 2017). In addition, continuous yield gain is paramount to feed the growing global population along with tolerance to climate change induced drought and heat stress and disease resistance combined with good agronomy can potentially improve the productivity to meet the future demands. The wheat biofortification breeding program at CIMMYT has made significant progress over the past 10 years focusing on improving grain Zn and Fe concentrations along with reducing phytic acid content for improved bioavailability in humans (Velu et al. 2020). Wheat is probably the crop with more genetic resources available in its secondary and tertiary genepools. Among these, genetic resources such as landraces, the old local varieties, and recreated synthetic hexaploid wheats are among the potential source for high Zn and Fe (Velu et al. 2011).

Large-scale screening of diverse genetic resources from CIMMYT germplasm bank and other sources has shown that there is a significant genetic variability for Zn and Fe content in some wheat genepools from primitive wheats, wild relatives, and landraces, indicating that Zn content is amenable to rapid breeding progress. Landraces and wild relatives of common wheat such as *Triticum spelta*, *T. dicoccon*, and *T. turgidum*-based synthetics that had the highest levels of Zn and Fe were used by the in targeted transfer using limited backcrossing into elite breeding lines (Guzmán et al. 2014).

Significant progress has been made in the past decade in transferring high-zinc alleles from these sources into elite breeding lines through targeted crossing and selection in relatively large segregating populations grown in Toluca and Ciudad Obregon environments in Mexico. Elite high Zn lines combining high Zn (and Fe), comparable yield potential, disease resistance, stress tolerance, and quality were identified; some released in India, Pakistan, Bangladesh, Nepal, Mexico and Bolivia already (Velu and Singh 2019).

2 Genetic Diversity and Targeted Breeding

Large-scale screening of diverse genetic resources from CIMMYT germplasm bank and other sources have shown that there is a significant genetic variability for Zn and Fe content in some wheat genepools from primitive wheats, wild relatives, and landraces.

In addition, screening of pre-breeding lines derived from elite and exotic parents showed large variation for grain Fe and Zn concentrations in wheat. Four entries (GID 7640819, 7254747, 7645287 and 7644342) showed more than 10 mg/kg Zn advantage and three entries (GID 7516893, 7644160, 7254747) showed about 5 mg/kg Fe advantage over the check (S. Table 4.1).

Table 4.1 Variance components for grain Zn, Fe and grain yield from stage 1 yield trials, Y18–19

Statistic	BLUP_Zn	BLUP_Fe	BLUP_GY
Heritability	0.81	0.74	0.83
Genotype variance	22.70	3.99	0.32
Residual variance	15.68	4.31	0.20
Grand mean	53.05	37.10	7.13
LSD	4.05	2.02	0.46
CV (%)	7.46	5.60	6.28

2.1 Current Breeding Approach

The targeted breeding focused on the simultaneous enhancement of high yield and enhanced Zn concentration has become the key objective after achieving success from the proof-of-concept approach. Each year about 400–500 simple crosses were made between elite high/moderate Zn lines, and between elite high Zn lines and best lines with normal Zn. Three-way crosses, or single back-crosses (BC1), are also made with a high-yielding parent. The BC1/F1Top and other segregating populations are shuttled between Obregon and Toluca field sites. In all generations, plants are selected for agronomic traits and disease resistance (all three rusts, *Septoria tritici* blight), 1–2 spikes from selected plants harvested as bulk, plump bold grains retained for advancing to next generation. Selected plants in the F4/F5 generations are harvested individually, selected for grain traits, and grown as F5/F6 small plots for phenotyping. Lines retained for agronomic traits and disease resistance are harvested, selected for grain characteristics and grain Zn and Fe concentration determined using XRF machine. High Zn carrying F5/F6 lines are advanced to stage 1 replicated yield trials at Obregón in the Zn-homogenized fields, which has shown good prediction of grain Zn in South Asia and other TPEs. Lines that yield similar or better than the checks in stage 1 yield trials are analyzed for grain Zn and Fe, and selected lines analyzed for end-use processing quality. Lines in stage 1 yield trials are also simultaneously phenotyped for resistance to Ug99 and yellow rust at Njoro, Kenya-off season, and the lines retained from Obregon trial again in the main season. Seed multiplication of retained lines then conducted in El Batan while they are also phenotyped for rusts and other diseases.

The competitive high Zn lines combined with key agronomic traits are distributed to NARS partners in South Asia and other TPEs. This led to identification and release of competitive high Zn varieties in TPEs. There are quite a few high Zn wheat varieties released in target countries of South Asia and beyond and adapted by >0.5 million smallholder farmers (Bouis and Saltzman 2017).

A recent yield data from the stage 1 trials from Ciudad Obregon showed about 1% average yield gain was achieved over the past 3 years while enhancing grain Zn concentration with +1–2 ppm annually (Figs. 4.1 and 4.2), suggesting a high probability of combining high yield with high Zn concentration. Although the mean yields of breeding lines derived from high Zn breeding pipeline and main breeding program were same, mean yield of "selected lines" with high Zn values were 4–6% lower than the mean of "selected lines" from main breeding program.

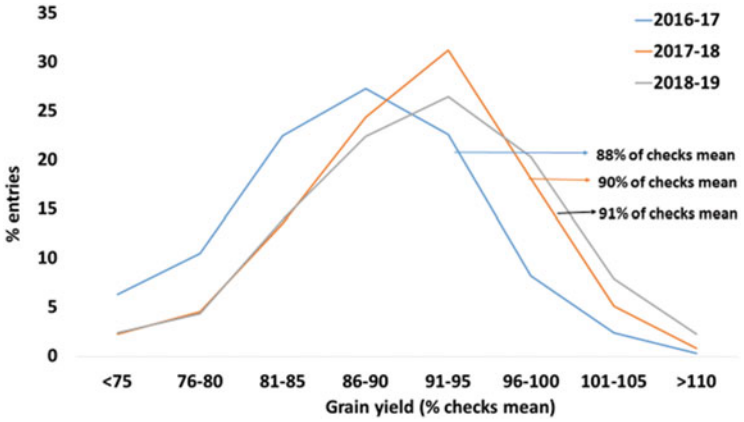

Fig. 4.1 Grain yield trends of wheat lines derived from three cohorts of Zn breeding pipeline evaluated in stage 1 replicated (3 reps) yield trials at Ciudad Obregón 2016–17, 2017–18, and 2018–19

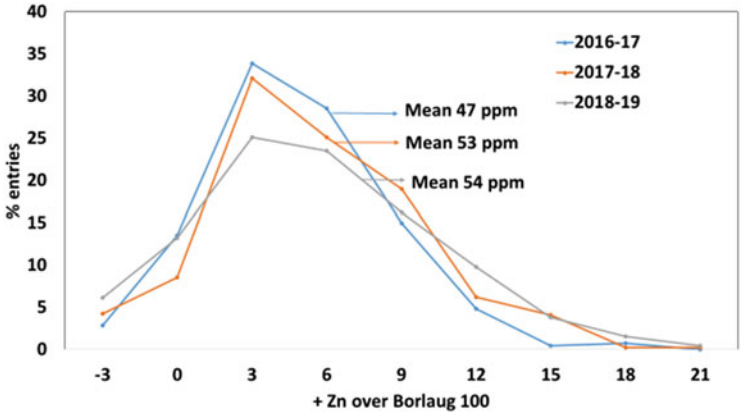

Fig. 4.2 Grain Zn concentration of wheat lines derived from three cohorts of Zn breeding pipeline evaluated in stage 1 replicated (3 reps) yield trials at Ciudad Obregón during 2016–17, 2017–18, and 2018–19

Moreover, the lack of association between grain yield and grain Zn will support their simultaneous genetic gain as realized in our current breeding pipelines (Velu et al. 2019).

3 Challenges and Opportunities

The major challenge over the next few decades will be to maintain the rates of genetic gains for grain yield along with increased grain Zn concentration as well as to close the yield gap of 4–6% between non-biofortified vs biofortified lines.

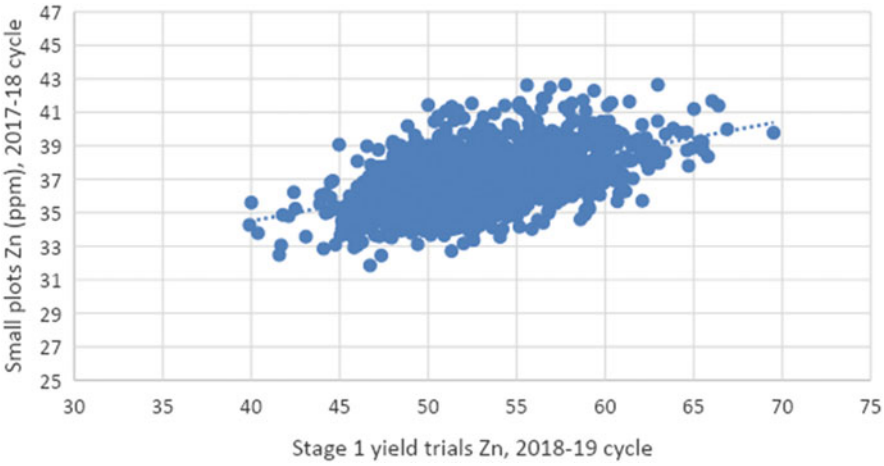

Fig. 4.3 Association between small plots vs stage 1 plots for grain Zn concentration, Y18–19

Therefore, to remain competitive, the performance of Zn-enhanced lines/varieties must be equal or superior to that of current non-biofortified elite lines/varieties, to ensure that smallholders will adopt them. Since both yield and Zn content are invisible and quantitatively inherited traits except few intermediate effect QTL regions identified for grain Zn, increased breeding efforts and new approaches are required to combine them at high frequency in CIMMYT's elite germplasm, ensuring that Zn levels are steadily increased to the required levels across the CIMMYT breeding pipeline.

The addition of Zn as a core trait will require a significant acceleration in the breeding cycle, expanding population sizes, phenotyping for Zn, yield testing and expanded land use, phenotyping for biotic and abiotic stresses, genotyping, molecular-assisted selection, and genomic selection.

In addition, heterogeneity within experimental plots for available soil Zn remains a bigger challenge. Our experimental fields at Ciudad Obregon have been optimized using soil application of Zn fertilizers over the years. Similar approaches will be followed in key sites in TPEs to optimize and improve the homogeneity for available soil Zn, which in turn helps in the identification of lines with better genetic potential to accumulate more Zn in grain.

Another challenge or limitation is the low correlation between small plots vs stage 1 yield trials ($R^2 = 0.25$) (Fig. 4.3). This may be due to disease pressure in the small plots, which were selected for rust resistance and agronomic performance when compared to yield trials evaluated for yield potential and then Zn and Fe content.

Table 4.2 Genetic and phenotypic correlations between grain yield and Zn, and grain yield and Fe, Y15–16 season and Y18–19 season

Genetic correlation			Phenotypic correlation		
Trait	Zn	Fe	Trait	Zn	Fe
First year yield trials Y15–16 season ($N = 1320$ lines)					
Fe	0.56		Fe	0.55	
GY	0.02	−0.17	GY	0.008	−0.14
First year yield trials Y18–19 season ($N = 1232$ lines)					
Fe	0.528		Fe	0.52	
GY	−0.06	0.05	GY	−0.076	0.04

3.1 Genetics and Variance Components

Although several QTL of moderate effect on grain Zn have been found in different germplasm sources, the genetic control of the trait appears to be as polygenic. In addition, grain yield and grain Zn are most likely independently inherited; due to the fact that no correlation has been observed between the two traits using multiple years of phenotyping results, and several studies at CIMMYT and partners have shown that moderately high heritability for Zn and Fe. The variance components from the Ciudad Obregón site showed genotypic (main) effects attributed to a larger share of total variation for grain Zn (61%) than the environment (39%), whereas multi-site analysis of an association genetics panel across locations in India showed 27% variation attributed to genotypic effects, 30% variation explained by genotype x environment interaction, and 43% by environment and error variance (Table 4.1).

Since no correlation between grain yield and Zn was found (Table 4.2), selection indices could be developed by giving economic weights to both the traits considering heritability and genetic variance estimates in target locations to develop an intra-population recurrent selection scheme through intercrossing well-defined parental lines, which should assist in capturing favorable additive effects to improve grain yield and grain Zn simultaneously. Also, Fe and Zn levels are highly correlated in wheat grain; this will likely result in significant improvements in Fe status as well.

3.2 Gene Discovery and Marker Development

Several genetic and QTL mapping experiments at CIMMYT and other published research have shown that inheritance of grain Zn (and Fe) is governed by small-to-intermediate-effect QTL of additive effects. The additive and additive x additive gene actions for the selection traits will allow the continuous addition of high grain Zn in high-yielding backgrounds by crossing the best elite lines from the current high Zn breeding lineage with the best elite high-yielding lines. Previous studies by CIMMYT and NARS partners have identified promising larger-effect QTL regions for increased grain Zn on chromosomes 2B, 3A, 4B, 5B, 6B, and 7B; and some QTL regions have a pleiotropic effect for grain Fe. Moreover, 2B and 4B QTL had a pleiotropic effect for increased thousand-kernel weight (TKW), suggesting that a

simultaneous improvement of grain Zn and seed size is possible (Cu et al. 2020; Liu et al. 2019; Srinivasa et al. 2014).

Based on our previous and ongoing studies, four promising QTL have been identified that have the potential to be used in forward breeding. These QTL showed a significant effect for grain Zn when combined in a favorable genetic backgrounds. Further progress is possible by accumulating the additive effect QTL dispersed across different lines into elite germplasm through marker-assisted breeding. We will implement forward breeding by taking advantage of the rapid generation advancement scheme to simultaneously introgress *QGzncpk.cimmyt-3AL* and *QZn. Across_4BS* in high Zn and normal zinc elite lines, further increasing Zn concentrations (Tiwari et al. 2016). This will aid the development of new parental sources for the Rapid Cycle Recurrent Selection (RCRS) pipeline to close the observed yield gap between high Zn and normal elite lines (Tables 4.3 and 4.4). Once the QTL have been introgressed, the developed markers associated with them can be included in the genomic prediction models as fixed effects, and the rest of the markers as random effects.

4 Future Breeding Approach: Novel Approaches for Mainstreaming

The moderately high heritability and significant positive association between environments for grain Zn concentrations under diverse target environments, the lack of associations between grain yield and grain Zn, combined with favorable associations between grain Fe and Zn densities, should permit efficient breeding for nutritious and high-yielding wheat varieties. Since both yield and Zn content are polygenic traits, increased breeding effort and new approaches are required to combine them at high frequency in CIMMYT's elite germplasm, ensuring that Zn levels are steadily increased across the CIMMYT breeding pipeline. This will be achieved by implementing increased population size, Zn screening of all the elite lines from the breeding program, and reducing breeding cycle times allowing simultaneous gains for Zn and grain yield together. This will allow all CIMMYT breeding lines distributed globally to exceed the yield level of current varieties and meet the Zn biofortification target of 36 ppm, about 40% above current levels, within next 10 years. The proposed approaches to mainstream grain Zn in wheat breeding involves:

- Increase the number of crosses and population size from crosses generated with high Zn elite parent with best elite parent and identify transgressive segregants for high yield and high Zn using traditional shuttle breeding pipeline (4 years scheme).
- Selection of best recipient elite parent for high yield and Zn from the bread wheat breeding pipelines and then cross with best high Zn elite parent and advance through Rapid Bulk Generation advancement (RBGA) using greenhouse and

Table 4.3 Performance Mexican landrace derivatives for grain Fe and Zn and grain yield potential

GID	Cross name	Grain yield (t/ha)	Zn (mg/kg)	+Zn over Borlaug100 (mg/kg)	Fe (mg/kg)	+Fe over Borlaug100 (mg/kg)
7254747	IG 41514/5/ SERI.1B//KAUZ/ HEVO/3/ AMAD*2/4/ KIRITATI/6/ FRET2*2/ SHAMA// KACHU	2.15	52	11	41	4
7516893	CPI8/GEDIZ/3/ GOO//ALB/CRA/ 4/AE. SQUARROSA (494)/6/KAUZ// ALTAR 84/AOS/ 3/PASTOR/4/ MILAN/CUPE// SW89.3064/5/ KIRITATI	2.52	47	6	43	6
7640819	CROC_1/AE. SQUARROSA (481)//KACHU/3/ BAJ #1	4.40	58	17	39	2
7644160	OAX93.15.1// WHEAR/ KRONSTAD F2004/7/SHA7/ VEE#5/5/VEE#8// JUP/BJY/3/F3.71/ TRM/4/ 2*WEAVER/6/ SKAUZ/PARUS// PARUS	5.19	47	6	42	5
7644342	SABUF/4/ ALTAR 84/AE. SQUARROSA (224)//CUPE/3/ CROC_1/AE. SQUARROSA (205)//F27202/8/ CNDO/R143// ENTE/MEXI_2/3/ AEGILOPS SQUARROSA (TAUS)/4/ WEAVER/5/ PICUS/6/TROST/ 7/TACUPETO F2001/9/KAUZ//	4.65	51	10	36	0

(continued)

Table 4.3 (continued)

GID	Cross name	Grain yield (t/ha)	Zn (mg/kg)	+Zn over Borlaug100 (mg/kg)	Fe (mg/kg)	+Fe over Borlaug100 (mg/kg)
	ALTAR 84/AOS/3/PASTOR/4/MILAN/CUPE//SW89.3064/5/KIRITATI					
7645287	68.111/RGB-U//WARD/3/FGO/4/RABI/5/AE.SQUARROSA (878)/6/ATTILA*2/PBW65//MURGA/7/BORL14	5.23	51	10	40	3
	Trial mean	5.02	44		35	
	Minimum	2.09	32		31	
	Maximum	6.15	58		43	
	Heritability	0.90	0.7		0.7	
	LSD ($P > 0.01$)	0.86	6.8		4.5	
	CV (%)	9.06	7.7		6.2	

field facility (3 years scheme) and look for best transgressive segregants with high yield and high Zn.

- Rapid cycle recurrent selection (RCRS) approach of high Zn elite x best elite crosses advanced in the greenhouse and GEBVs calculated for the parents and progenies and progeny lines with highest GEBV for Zn and yield will be recycled as a population improvement approach (2 years recycling time). Though the mean levels of Zn and yield potential among the populations increased over 2–3 cycles of a recurrent selection scheme, the resulting progenies will have to be fixed for disease resistance and processing quality to ensure release in targeted countries.

In order to achieve above-mentioned breeding schemes we are in the process of generating genotypic data for high Zn wheat lines and training populations specific for biofortification breeding being generated. Prediction models developed using novel statistical genetic models (ex. GBLUP) incorporating all the available genomic and phenomic information will be validated and utilized in the RCRS breeding pipeline for selection of potential parents and progenies with high breeding values for Zn and grain yield, to accelerate higher genetic gains for grain Zn and grain yield simultaneously. For instance, genomic predictions for Zn and Fe were moderately high ($r = 0.4$–0.6) across locations in Mexico and India using the association mapping panel from biofortification program (Velu et al. 2014). Therefore, GS models for these traits could also be built for selecting parents.

Table 4.4 Heritability and variance components for grain Zn and Fe across locations, eighth HPYT

Trait	Country	Entry variance	Residual variance	Grand mean	LSD	CV	Heritability
Grain Zn (ppm)	Obr-Bed-5Irr	2.51	1.73	26.8	2.72	4.97	0.74
	Obr-Bed-2irr	6.69	4.17	34.0	4.34	6.24	0.76
	PARC, Islamabad	5.49	9.40	34.2	6.39	9.15	0.54
	PARC, Faisalabad	5.51	8.84	29.3	6.34	10.58	0.56
	PAU, India	8.69	14.54	28.5	8.22	14.11	0.54
	Karnal, India	20.46	18.48	32.1	9.25	14.11	0.69
	Gurdaspur, India	1.57	10.97	32.3	6.71	10.17	0.22
	Hisar, India	18.12	10.61	44.1	6.52	7.23	0.77
	Across locations	3.70	11.50	32.5	2.90	4.60	0.78
Grain Fe (ppm)	Obr-Bed-5Irr	9.63	3.56	32.6	3.83	5.76	0.84
	Obr-Bed-2irr	17.26	7.71	42.7	6.07	6.97	0.82
	PARC, Islamabad	7.25	10.08	34.8	6.97	9.80	0.59
	PARC, Faisalabad	16.82	8.74	37.8	6.43	8.31	0.79
	PAU, India	2.08	2.49	32.4	3.32	5.02	0.62
	Karnal, India	11.33	3.74	37.3	4.04	5.30	0.86
	Gurdaspur, India	1.64	4.50	35.5	4.33	5.97	0.42
	Hisar, India	7.08	2.50	38.9	3.27	4.12	0.85
	Across locations	5.10	6.60	37.0	2.40	3.30	0.88

The options are to (a) use the trait pipeline to introgress disease resistance genes in high-value elite lines/parents, (b) go through a breeding cycle using the rapid bulk generation advancement scheme (RBGA), or (c) use CIMMYT's current shuttle breeding pipeline. In addition, to accommodate the increased number of lines for high Zn pipeline, large area is being optimization of available soil Zn at the Ciudad Obregon fields.

In addition, the wheat biofortification program requires fast, accurate, and inexpensive methods of identifying nutrient dense genotypes. The energy-dispersive X-ray fluorescence spectrometry (EDXRF) has been standardized to screen Zn and Fe concentrations in whole grain wheat samples (Paltridge et al. 2012). The EDXRF

provides grain Fe and Zn with highest accuracy and precision with simple and fast sample preparation for the analysis of grain micronturients.

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Barley Biofortification

5

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Abstract

Barley is the fourth largest cereal crop in the world in terms of production, which is commonly used for animal feed and malting globally. However, it is an important staple food in dry areas of Asia and Africa. Currently, it is being considered as a functional food because of its medicinal value in reducing the risk of a number of health problems. It is a very good source of soluble fiber and the nutritive value is similar to the main cereals. Beta glucans, the major components of endosperm cell walls are considered very important from barley food usage point of view in decreasing the blood LDL cholesterol and controlling the blood glucose levels. Biofortification efforts gaining attentions to increase the nutrients contents in the grain using genetic or biotechnological and agronomic, interventions to further improve its nutritional value. There is need to biofortify barley with minerals (iron, zinc and selenium), vitamins, essential amino acids, and bioactive compounds with health beneficial activities. Along with the increase in the content, improving the bioavailability is also equally important. The chapter deals with the efforts made to improve barley in terms of minerals, vitamins, and bioactive compounds and also with the future prospects of barley biofortification.

Keywords

Biofortification · Barley · Anti-nutrients · Minerals · Vitamins · Beta glucan · Bioavailability

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1 Introduction

Barley (*Hordeum vulgare* L.) is one of the first domesticated crops and ancient among cereals with a significant role in the development of human civilization. The cultivated barley is a diploid species with $2n = 14$ chromosomes and large genome size (>5.1 giga bases) consisting of highly repetitive sequences, almost 12 times the size of rice genome (Bennett and Smith 1976; IBGSC 2012). Barley is grown over diverse eco-geographical environmental conditions as compared to other crop species, because of its hardiness to environmental variations. Barley is often considered the only possible rainfed cereal crop under low input and stressful environments, like drought, heat, and cold. This adaptability to extreme and marginal conditions has led to widespread cultivation of this cereal throughout the world (von Bothmer et al. 1995). The range of barley cultivation is from the tropics to high latitudes (>60°N) in Iceland and Scandinavia as well as in high altitude up to 4500 meters above sea level (masl) in the Himalayas (von Bothmer et al. 2003; Ceccarelli et al. 2008). In terms of total production, barley ranks fourth in the world among cereals after wheat, maize, and rice (FAOSTAT 2020). It is grown by nearly 100 countries on about 49 million hectares (ha), producing about 146 million tones with a yield of 29.9 q/ha (Table 5.1). Europe is the largest in terms of the barley area (49.8%) and production (61.1%) followed by Asia and Africa. In terms of productivity also Europe is highest with 3.7 t/ha among all continents to closely followed by America (3.6 t/ha). Globally, the area under barley cultivation decreased from 80 million ha in the 1980s to less than 50 million ha in 2018 (FAOSTAT 2020).

The major barley importing countries during 2018 include Saudi Arabia followed by China, Iran and Netherlands, while France, Australia, Russia, Ukraine, Argentina and Canada are major exporters (Table 5.2).

These figures, however, may fluctuate annually in the North African countries based on the adverse effect of drought, on barley production as mainly it is rainfed in the region. Under variable climatic conditions within the growing season, such as drought, heat, or cold, barley gives comparably higher yields than other small grain cereals. Being one of the most widely adapted crops, the barley germplasm pool has the potential to contain enough genetic diversity to breed for adaptation to different environmental conditions. Moreover, the ample barley germplasm resources available worldwide (Bockelman and Valkoun 2010), including wild relatives, likely to contain beneficial allelic variation that new molecular breeding technologies can

Table 5.1 Continent wise area, production, and productivity of barley

Regions	Area (mha)	Production (mt)	Yield (Q/ha)
Africa	4.66	6.67	14.3
America	4.73	17.03	36.0
Asia	10.71	21.18	19.8
Europe	24.11	89.77	37.3
Oceania	4.25	10.29	24.1
World	48.73	145.86	29.9

Source: FAOSTAT (2020)

Table 5.2 Major global traders of barley grains

SN	Country	Export		Country	Import	
		Quantity (t)	Value (000$)		Quantity (t)	Value (000$)
1	France	6,196,232	1,325,960	Saudi Arabia	7,656,637	1,032,636
2	Australia	6,123,369	1,392,423	China	6,815,355	1,690,391
3	Russian Federation	5,441,666	1,024,203	Iran	2,648,611	602,794
4	Ukraine	3,597,474	681,924	Netherlands	2,202,270	480,800
5	Argentina	2,587,696	537,089	Belgium	1,747,592	386,189
6	Canada	2,238,693	527,399	Germany	1,280,020	292,532
7	Germany	1,863,190	377,659	Japan	1,264,034	348,387
8	Kazakhstan	1,754,980	293,537	Jordan	863,578	197,367
9	Romania	1,332,133	278,102	Libya	692,226	165,962
10	United Kingdom	838,405	191,333	Turkey	655,988	150,782

Source: FAOSTAT (2020)

exploit (Newton et al. 2011). Owing to its vast morphological and environmental adaptability, various types of barley (winter, spring, two-row, six-row, awned, awnless, hooded, covered, naked, malting, feed, and food types) are grown throughout the world.

2 Barley Utilization

Barley has considerable economic importance both in agriculture and industry in many countries. Globally, around 55–60% of barley production is used for feed, 30–40% for malt, 2–3% for food, and 5% for seed (Ullrich 2010). Around 3.7% of total barley production is used as a human food annually worldwide, but in some countries like in Morocco, Ethiopia, and Eretria, barley is used as food as high as 60% of total production (Newman and Newman 2006). Apart from this, some countries in West Asia like Iran use barley soup as a regular part of the diet. The medicinal value and the health benefits of barley have been enumerated in the ancient literature throughout the world and is often called *the king of grains*. In ancient Rome, gladiators were called *hordearii* or "barley men" because of their preference for highly nutritious barley grain, which is considered as a source of strength and stamina for athletes and laborers.

Use as a calorie food source for human consumption is mainly confined to marginal areas with problematic soils and scanty rainfall (Grando and Macpherson 2005). It is the major dietary source for ruminant and non-ruminant livestock, poultry, and fish. Generally, the feed barley varieties yield more (10–20%) than the malt barley varieties (Blake et al. 2010). This is because the malting industry prefers barley kernels of similar size, which allows for a more uniform malting process. Uniform kernels are easier achieved in a two-row variety, where seeds are

more equally spaced than in six-row varieties, where due to crowding of seeds in certain positions are larger than seeds in other positions. Recent research regarding dietary composition in food barley has renewed interest in this end-use, confirming the health benefits of barley in human diets (Brockman et al. 2013; Sullivan et al. 2013) through more soluble dietary fiber mainly beta glucan content and other health beneficial phytochemicals than other food cereals. Barley is a common diet for diabetic people due to lower glycemic index. In comparison to other cereal crops, barley has a better fodder value including grain and straw. In most of the developed countries, barley straw is used for animal bedding, whereas it is used as animal feed in the developing countries, in addition to the grazing use in most of the West Asia and North Africa.

The economically important cereal species such as maize, rice, wheat, and barley are being utilized as major sources of human diet directly or indirectly (through livestock). Historically, owing to its rich dietary fiber and readily available energy, barley was utilized by the Roman gladiators, who were also called as "hordearii" (Andrew 2008). Although globally the major utilization of barley is for feed and malting purposes, because of its nutritional value barley is consumed as a staple food in North and Sub-Saharan Africa (SSA), Central Asia, and South-West Asia. Barley is one of the earliest domesticated cereal crop and ancient evidences show its importance in human diet. Currently, it is a major part of food grain in dry areas of developing world especially in Asia and Africa, where it is used for a variety of products ranging from barley cookies, Couscous (north Africa), Angera (Ethiopia and around in east Africa), Soup (Iran), Dalia & flacks (South Asia) for direct consumption in addition to mixing with other grains like multi-grain flour to improve the nutritional value (Grando and Macpherson 2005; Narwal et al. 2017). The continuous growth in the human population has created necessity for development of crops with higher yield to ensure adequate amounts of food. This approach has resulted in the reduction of grain quality including the decline in protein and mineral nutrient concentrations resulting in the so-called hidden hunger especially in the developing countries.

It has been predicted that over 2 billion people worldwide suffer from iron, zinc, and/or other multiple micronutrient deficiencies (WHO 2016; Black 2003). The problem of micronutrient malnutrition is most prevalent in low- and middle-income developing countries. In Africa, the estimated risk for deficiency of calcium, zinc, selenium, iodine and iron is 54%, 40%, 28%, 19% and 5% of the continental population respectively (Joy et al. 2014). Iron deficiency is the main cause of anemia (50% of total cases) which in turn is associated with poor pregnancies, impaired cognitive development, reduced immunity, and work productivity (WHO 2017). The FAO has estimated that out of 792.5 million malnourished people in the world, 780 million people live in developing nations (McGuire 2015).

3 Barley as Healthy Food

Barley is an important crop with widest range of cultivation from cold deserts/highlands of Tibet to the sea levels and often considered as the last crop before hot Sahara deserts. It has lesser inputs requirement also as compared to other cereals and thus can be grown with relatively lesser resources *vis-a-vis* other cereals. Barley matures earlier and has evolved to use less heat units than other crops. Therefore, barley is currently being referred as "crop for climate change" for its capacity to adapt with wider climatic conditions from extreme cold to hot environments as well as a big range of cropping days differences, in addition to its capacity to perform under low input conditions. Among micronutrients deficiency, zinc (Zn) and iron (Fe) are the most prominent elements because of which over 2 billion people are affected across the globe. Deficiencies of elements, specifically Fe, Zn, magnesium (Mg), phosphorous (P), potassium (K), selenium (Se), and copper (Cu) are the major causes of over 65% childhood death worldwide (Welch and Graham 2004). They used the term "hidden hunger" to describe the deficiency of multiple elements (micronutrients) and malnutrition. The deficiencies of Zn and Fe alone are considered two major factors for several nutritional-related human disorders or diseases worldwide and pose greater threat to human health specifically in developing countries (WHO 2002). Barley being a nutritionally dense food consists of a balance of complex carbohydrates, proteins, and an assortment of vitamins and minerals (Table 5.3). Barley is considered as one of the high-energy, heart-savoring healthy food (Gyawali et al. 2019).

It has been clinically proven as one of the preventive options in cardiovascular diseases and management of type II diabetes. The glycemic index of barley is also very low (Atkinson et al. 2008; Thondre and Henry 2009) as compared to other cereals (Table 5.4) and hence consumption of whole grain barley can be placed in the category of nutraceutical cereals and has been shown to help in the maintenance of body weight. Barley is a store house of dietary fibers which are the non-digestible

Table 5.3 Nutrient composition of hulled barley

Nutrient	Unit	Value/ 100 g	Nutrient	Unit	Value/ 100 g
Energy	kcal	354	Magnesium	mg	133
Protein	g	12.48	Iron	mg	3.6
Total lipid	g	2.3	Zinc	mg	2.77
Carbohydrates	g	73.48	Vit A	IU	22
Fiber	g	17.3	Vit E	mg	0.57
Total sugar	g	0.8	Thiamin	mg	0.646
Fatty acids, total saturated	g	0.482	Riboflavin	mg	0.285
Fatty acids, total mono unsaturated	g	0.295	Niacin	mg	4.604
Fatty acids, total poly unsaturated	g	1.108	Vit B6	mg	0.318
Calcium	mg	33	Folate	µg	19

National Nutrient Database for Standard Reference 1 Release April, 2018

Table 5.4 Glycemic index of some of the cereal and cereal food products

Food	Glycemic index (glucose = 100)
Barley	28 ± 2
White wheat bread	75 ± 2
Specialty grain bread	53 ± 2
Chapatti	52 ± 4
White rice, boiled	73 ± 4
Brown rice, boiled	68 ± 4
Sweet corn	52 ± 5

Source: Atkinson et al. (2008)

Table 5.5 Range of grain beta glucans in barley, oats, and wheat grain

Crop	Beta glucan (%) (mean and range in parenthesis)
Spring barley	4.16 (1.86–5.37)
Oats	3.49 (1.73–5.7)
Spring wheat	0.48 (0.19–0.67)

Source: Havrlentova and Kraic (2006)

carbohydrates. It contains both soluble and insoluble fiber and total dietary fiber ranges from 11% to 34% while soluble dietary fiber ranges from 3% to 20%. Soluble fibers include beta-glucan, pectin, and some hemicelluloses and have many health implications. Soluble fiber is reported to be effective in lowering blood cholesterol levels and therefore can help in reducing the risk of heart disease. It has been shown that the inclusion of barley flour increased the beta glucan content of wheat chapattis considerably (Narwal et al. 2017). The major component of barley imparting its health properties is the soluble fiber mixed linkage beta glucans. Barley (besides Oats) is one of the unique cereals having higher content of beta glucans as compared to other cereals (Table 5.5).

It also slows down the absorption of sugar and can reduce the risk of type 2 or non-insulin-dependent diabetes. There is a big advantage with barley β-glucan. In other grains, most of the β-glucan is removed when the bran layer of the grain is removed, but, in barley, β-glucan is found throughout the entire kernel and therefore even refined products contain a substantial amount of β-glucan. On average, pearled barley has very low glycemic index of 25 and a comparatively low glycemic load of 11, which is an excellent combination. Studies have shown that barley is especially effective in the prevention and treatment of diabetes. Barley and malt are now gaining renewed interests and are being used as ingredients of many functional foods because of the high content of soluble fiber β-glucan and many bioactive compounds with antioxidant activity. In developed countries, people have become more health conscious and are now adopting functional foods that can provide additional health benefits besides the basic nutrition. Barley is one of the top functional food utilized in developed countries in many bakery products and other recipes.

4 Phytochemicals and Antioxidant Activity in Barley

Besides providing basic nutrition, barley is also a store house of a number of phytochemicals. These substances have a number of biological functions and therefore called the bioactive compounds. Important groups of phytochemicals with great beneficial nutritional and health effects are phenolics, carotenoids, tocols, lignans, phytosterols, folate, and β-glucan (Table 5.6). The bioactive phytochemicals in

Table 5.6 Content of bioactive compounds and antioxidant activity in barley

Composition	Kernel position	Mean ± SD	Range
β-Glucan (%)	Whole grains	4.61 ± 0.45	2.40–11.00
Resistant starch (%)	Whole grains	3.63 ± 2.32	0.2–24.0
Arabinoxylan (%)	Endosperm	0.67 ± 0.06	0.53–0.90
	Barley bran	4.66 ± 3.35	1.97–8.42
	Whole grain flour	1.31 ± 0.73	0.70–2.13
Polyphenols (mg/100 g)	Whole grain	231.61 ± 34.26	150.0–300.0
	Barley bran	421.84 ± 24.46	376.1–443.5
	Whole grain flour	140.41 ± 10.21	129.9–160.7
Phenolic acids (mg/100 g)	Whole grains	414.70 ± 32.86	336.29–453.94
Total flavones (mg/100 g)	Whole grains	80.64 ± 17.15	37.93–236.91
Flavonoids (mg/100 g)	Whole grains	12.51 ± 10.14	6.20–30.08
Catechin (mg/100 g)	Whole grains	2.25 ± 0.94	0.90–4.27
Quercetin (mg/100 g)	Purple grains	3.51 ± 2.24	2.00–6.08
Kaempferol (mg/100 g)	Whole grains	3.66 ± 14.87	1.27–6.31
Myricetin (mg/100 g)	Whole grains	11.07 ± 22.25	0–73.30
Total alkaloid (mg/100 g)	Whole grains	25.96 ± 1.41	6.36–44.63
Total anthocyanin (mg/100 g)	Whole grain	35.50 ± 23.82	4.9–103.7
	Barley bran	256.05 ± 137.67	158–353.4
	Refined flour	39.15 ± 25.67	21.0–57.3
Proanthocyanidin (mg/100 g)	Whole grains	6.97 ± 3.84	1.58–13.18
Total tocols (mg/100 g)	Whole grains	5.85 ± 3.51	0.85–12.49
Antioxidant activity (%)	Whole grains	41.55 ± 7.82	24.10–82.00
GABA (mg/100 g)	Whole grains	8.00 ± 3.92	0.10–30.67
Folates (mg/100 g)	Whole grains	71.24 ± 16.62	51.8–103.3
Phytosterols (mg/100 g)	Whole grains	91.13 ± 21.14	76.1–115.3
ABTS-IR50 (g/L)	Grain alkaline extract polysaccharide	2.12 ± 0.35	1.74–2.84
ABTS-TEAC (mg/g)		8.94 ± 1.34	6.50–10.61
FRAP (μmol/g)		90.58 ± 21.61	51.1–131.1
ORAC (μmol/g)		380.28 ± 161.24	147.81–652.46
ABTS-IR50 (g/L)	Grain water extract polysaccharide	10.59 ± 1.69	7.41–13.43
ABTS-TEAC (mg/g)		1.79 ± 0.31	1.37–2.49
FRAP (μmol/g)		32.14 ± 9.35	15.80–41.80
ORAC (μmol/g)		206.49 ± 106.83	71.49–396.57

Source: Zeng et al. (2020)

barley have been recently reviewed by Idehen et al. (2017). Barley grain polyphenols include phenolic acids, flavonoids, tannins, and proanthocyanidins and are concentrated in the hull, testa, and aleurone. The content of various phenolic compounds and antioxidant activity in barley are significantly affected by the growing location, the growth year, and the genotype. These phenolics exist in free, esterified, and insoluble-bound. Most phenolic acids exist in the bound form with other grain components such as starch, cellulose, beta glucan, and pentosans. Vitamin E is the major lipid-soluble antioxidant for human health and barley contains all eight tocol vitamers, which are usually not complete in some cereals. Phenolics are the predominant compounds in cereals like barley which contribute to the antioxidant potential due to the presence of an aromatic phenolic ring that can stabilize and delocalize the unpaired electron within the aromatic ring. They are believed to act mainly as free-radical scavengers, and/or chelators of transition metals. Barley grains contain much greater amounts of phenolic compounds than other cereal grains and has been found to have high antioxidant activity than other common cereals such as wheat and maize (Fig. 5.1). The antioxidant potential of barley has been reported by many researchers using different antiradical systems. The colored barley types have high anthocyanin content which are health-promoting flavonoids. Purple and blue barley groups contain higher average contents of anthocyanins than black.

Natural antioxidants present in various foods can improve the redox status in the biological systems and reduce the risk of aging related health problems including

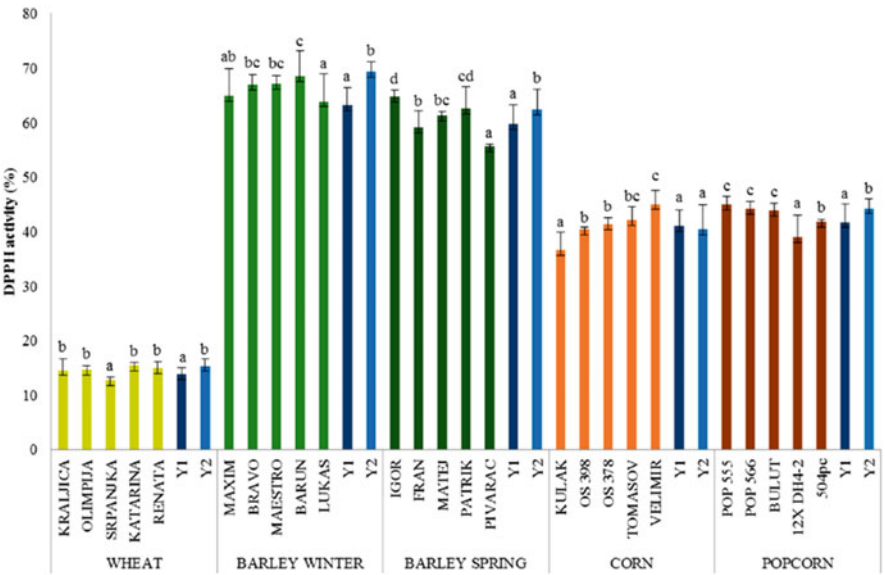

Fig. 5.1 DPPH radical scavenging activity (%) in cereal grains. Mean ± SD of 2 years and triplicate extraction ($n = 6$). Bars with different superscript letters (a–d) are significantly different ($p < 0.05$). (Adapted from Horvat et al. 2020)

cancer and heart diseases. A wide range of bioactive nutrients and their pleiotropic physiological effects make barley an ideal grain, raw material, and ingredient for the development of functional foods.

Barley can serve as an excellent dietary source of antioxidants with antiradical and antiproliferative potentials for disease prevention and health promotion. Barley consumption has been associated with lower total and serum cholesterol, improved postprandial glucose and insulin response, and reduced heart disease and colon cancer. Once absorbed, these phytochemicals are metabolized and may contribute through both direct and synergistic pathways to impact health via anti-inflammatory, antioxidant, and/or anti-proliferation effects.

For many food products, whole grains undergo varying degrees of processing that may lead to an improvement in the bioavailability of its constituent phytochemicals. The outer structure of the grains, including the pericarp seed coat and aleurone layer, generally contain much higher phytochemical concentrations than the germ and endosperm compartments, and the ultimate bioavailability of these phytochemicals may depend greatly on the degree and manner in which the grain is processed before consumption. Few studies have examined the bioavailability of phenolic acids and polyphenols from oats and barley in humans. To date, no clinical trial has examined the bioavailability of phenolic acids in barley. In addition, since specific studies on health effects of phytochemicals in barley are limited, it is worthwhile to further study the efficacy and the underlying molecular mechanisms of barley phytochemicals, thereby promoting the use of barley as a functional food.

In general, milling and pearling processes affect the distribution of phenolic, compounds and thus antioxidant properties vary among the milling fractions. During pearling, both the phenolic content and the antioxidant activity decrease from outer to the inner parts of the kernel. Thus, barley fractions with varying concentrations of phenolic compounds and antioxidant potentials can be produced through controlled pearling process. Malting process allows better release and/or extraction of phenolic compounds. In beer, 70–80% of the phenolic constituents originate from malted barley. Polyphenols and phenolic acids present in malt are natural antioxidants, capable of delaying, retarding or preventing oxidation processes and therefore are thought to have a significant effect on malting and brewing as inhibitors of oxidative damage. Other processes like sprouting, germination, fermentation, and sand roasting also result in a significant changes in antioxidant activity.

Suitable processing technologies can enhance the bioavailability of the bound phenolic compounds. This can be achieved primarily through particle size reduction, structural breakdown of cereal matrices, and their release from cereal matrices. Extrusion cooking and thermal treatments of cereal grains may affect bioavailability of phenolic compounds either positively or negatively as high temperatures may cause decomposition of heat-labile phenolic compounds or result in polymerization of some compounds during high-pressure extrusion cooking. In cereal grains like barley, the bioavailability of phenolic compounds depends on the grain type and the processing method and the conditions used. The mechanical processing and bioprocessing have positive effects on the bioavailability of grain phenolic

compounds. Thus, the use of a proper combination of these two processing methods is worth investigating in future.

5 Biofortification to Improve Nutritional Quality of Barley Food and Feed

Modern agriculture is now prioritizing to produce food crops that are rich in micronutrients to help in fighting "hidden hunger" especially in poor developing countries, where the diet of most of the population consists of starch-rich and nutrient-poor staple food crops. Further, plant also needs adequate supply of the nutrients for its own growth to achieve higher yields. Thus, re-biofortification of the staple food crops represents a worthwhile approach (Garg et al. 2018). The process of increasing the content and/or bioavailability of essential micronutrients in food crops during plant growth either through genetic or agronomic approaches is called biofortification (Bouis et al. 2011). Biofortification is one of the widely accepted strategies and considered a sustainable approach of improving element concentration in economic plant parts, their bioavailability in food, and resolving mineral malnutrition in human (Welch and Graham 2004; Singh et al. 2005; Uauy et al. 2006). It is perhaps the most cost-effective method of mitigating element deficiencies in developing countries, specifically Zn and Fe. Biofortification mainly focuses on staple starchy crops (rice, wheat, maize, sorghum, millet, sweet potato, and legumes), as they dominate the human diets in poor developing countries. This can be the most feasible means of reaching malnourished populations which have limited access to diverse diets, micronutrient supplements, and the commercially fortified foods. The desired concentration of micronutrient for a specific staple crops can be calculated based on the recommended daily allowances of different micronutrients, per capita consumption of staple crops, nutrient losses during food processing and preparation, and the estimated bioavailability (Bouis et al. 2011). The main focus of the biofortification programs is on the essential micronutrients (vitamins or minerals) which are required in amounts less than 1 mg/day. These micronutrients are essential for several biological processes and serve as metabolic precursors or enzyme cofactors and their deficiency may result in characteristic diseases. Thus, a biofortification strategy for staple food crops should have the potential to improve the delivery of the micronutrients to human diets thereby improving the nutritional status of vulnerable communities of the world. Biofortification of micronutrients can be achieved through agronomic approaches, conventional breeding of food crops or transgenic approaches. Agronomic approach involves the application of mineral fertilizer either to the soil or foliage to increase the content of mineral elements in the edible parts. Conventional breeding involves the crossing of genotypes with high mineral or vitamin levels over several generations to produce plants with desired nutritional and agronomic traits. When a particular nutrient does not exist naturally in a food crop or it cannot be bred effectively in sufficient amounts through breeding, then transgenic approaches can play an important role which may lead to micronutrient gains beyond those available through conventional plant breeding. But, even if

a transgenic line is developed, the transgenes should be stably inherited, and this may further require conventional breeding for many years. Also, the incorporation of this trait into the popular high-yielding varieties may need several years. Moreover, many developing countries lack the basic legal framework required for the release and commercialization of the transgenic crops (Saltzman et al. 2013).

The international initiatives like Harvest Plus program were started in 2003, to deliver biofortified staple crops which have the potential to increase the concentrations and/or bioavailability of essential micro nutrients in human diets. The Harvest Plus consortium has suggested an absolute target for the additional Zn and Fe in biofortified crops of between 30 and 40% of the estimated average dietary requirement for humans (White et al. 2009).

5.1 Genetic Variability in Mineral Content

Barley is considered a model crop for biofortification studies. Several barley genes (NAS, NAAT, DMAS, IDS2, and IDS3), responsible for higher element uptake specifically Fe and Zn, are used for biofortification in other crops such as rice (*Oryza sativa* L.) which lacks these genes (Takahashi et al. 1999; Nakanishi et al. 2000; Kobayashi et al. 2001; Masuda et al. 2008, 2009). However, in the past, biofortification of elements were focused to Fe and Zn, while other elements were less studied in barley. Birsin et al. (2010) reported the distribution of multiple element in different plant parts (leaf, stem, and spikes) during plant developmental stages including vegetative and reproductive stages. Micronutrients were redistributed into the grains during late reproductive stages in barley (Birsin et al. 2010). Therefore, the first step toward the exploitation of biofortification of elements in barley is to assess the status of multiple element concentrations in the grains and their interrelationship during uptake into barley grains. The availability of wide genetic variability within a target gene pool is the most important pre-requisite for breeding crops with a specific trait. In most of the cereal crops, genetic variation is available in the concentrations of mineral elements. Iron and zinc concentration in cereal grain can vary 1.5–4.0-fold among genotypes depending on the available genetic diversity. The breeding for Fe and Zn biofortification in cereal grains is somewhat complex because various physiological processes govern their concentration in the grain. In barley, genetic variation in the content of minerals has been reported by various studies across the world. Wild barley (*H. spontaneum*) is reported to have higher genetic variability for mineral concentrations as compared to the cultivated barley. The high Zn and Fe concentration in ICARDA germplasm was mainly contributed by either wild relative (*H. spontaneum* [Hss]) and/or landraces of *H. vulgare* (Hv). Specifically, H.spont.41-3, H.spont.41-5, and H. spont.38-3 were frequently used in crossing block of low input barley breeding program as major sources of drought tolerance and high Zn and Fe concentration in grain (El-Haramein and Grando 2010; Lakew et al. 2011). Barley genotypes with high multiple mineral element concentrations have been identified in ICARDA

Table 5.7 Genetic variation available in the Fe and Zn content of barley

Barley source	Iron	Zinc	Reference
ICARDA, Morocco	21.9–66.2	10.4–338.1	Gyawali et al. (2019)
Japanese barley	24.6–63.3		Ma et al. (2004)
American barley	21.0–83.0		
Barley grains registered in Russian Federation	24–79	6–33	Bityutskii et al. (2017)
Wild barley (*H. spontaneum*)	10.8–329.1	66.3–493.9	Yan et al. (2012)
Barley landraces from Ethiopia and Eritrea		30.7–48.5	Mamo et al. (2014)

germplasm, which can be utilized effectively for biofortification programs (Gyawali et al. 2019). The results of these studies have been summarized in Table 5.7.

Minerals are non-homogenously distributed within the grain and the highest concentrations of many of the elements are found in the outer husk and the aleurone layers. Most of the cereals are consumed only after milling, polishing/pearling which removes major portion of mineral elements from the human diet and the genotype determines the extent of these losses. The distribution of mineral elements like iron and zinc within the grains is affected by grain morphology traits like size of the grain and embryo and the thickness and number of the tissue layers. Spatial distribution studies in barley by grain dissection and l-PIXE analysis of contrasting lines has indicated that differences in grain zinc accumulation occur in all parts of the grain including endosperm. In rice, maize and barley, the concentration of Ca, Mg, Zn, Fe and Cu is related to the number of aleurone layers which in turn is cultivar dependent (Detterbeck et al. 2016). Thus, the genetic variations in the spatial distribution of minerals within the grain should be utilized in planning different biofortification strategies.

Genetic variation in the mineral content in cereal crops can be enhanced by different genetic processes. New genetic variation can be introgressed into the gene pool of modern elite crops from wild progenitors (*Hordeum spontaneum*). Introgression of *Gpc-B1* locus from wild emmer (*Triticum turgidum* ssp. *dicoccoides*) into cultivated wheat through chromosomal substitution is a successful example. This locus has been shown to have a positive impact on the content of grain Fe, Zn, Mn, and proteins without any significant negative impact on yield. Many studies have reported wide phenotypic variation in wild barley for a number of agronomic traits, but their utilization in biofortification programs is very limited (Wiegmann et al. 2019). Further, in order to enhance the genetic variation in the micronutrient content of barley, a wild barley population, HEB-YIELD which is selected subset of the nested association mapping (NAM) population HEB-25 was developed. A huge variation was observed in the nutrient concentration in grains. Some lines even showed >50% higher grain iron, zinc, and protein content in comparison to the recurrent parent. These lines can be used directly in crossing programs and also for the identification of genes controlling the content of different micronutrients in the barley grain. The genomic regions like those on the short arm

of chromosome 5H indicate that there are chances of wild barley possessing alleles which can increase the nutritional value without any yield penalty and may function as correlation breakers. These promising alleles from wild barley could be ultimately introgressed into the elite crop material. Additionally, the expression of effective alleles can be regulated by genetic engineering (Wiegmann et al. 2019).

5.2 Genetic Variability in Grain β-Glucan Content

Grain beta glucan content is mainly determined by genetic factors. The genotypes intended for food barley are expected to have higher grain beta glucan while for malt purpose barley these should have low grain beta glucan. There is wide variation in grain beta glucan content, Nishantha et al. (2018) studied the grain β-glucan in wild and cultivated barley collected from several parts of the world and in case of wild barley accessions it ranged from 3.26% to 7.67% and approximately 60% of accessions were in the range of 4.0–6.0%. β-Glucan content in case of cultivated barley varieties ranged from 2.68% to 4.74% and approximately 90% of cultivars fell in the range of 3.0–5.0%. In a study conducted in sub-tropical climates of India, the grain beta glucan ranged from 2.9% to 7.1% (db) (Kumar et al. 2017a, b). Zhang et al. (2002) analyzed the β-glucan content of barley cultivars from different areas of China, Canada and Australia, grown in a multi-location trial in China. The cultivars originating from China, β-glucan content ranged from 2.98% to 8.62%. The hull less barley from Tibet had the highest values of beta glucan. Blakely and Harasymow (2010) also stated that genotype is the major contributor to the beta glucan content in a study conducted in Australia. Besides the nature of grain coverage, i.e., hulled or naked grain, the ear type also influences the grain beta glucan content. In one study conducted with several genotypes of two row and six row barley, the two rowed barley had more beta glucan as compared to six rowed.

5.3 Genetic/Transgenic Approach to Biofortification

Biofortification of food crops is considered the cheapest and is easily accessible to poor people (Welch and Graham 2004) among the three strategies (biofortification, food fortification, and element supplement) for resolving nutritional deficiency. The aim of genetic biofortification is to develop plant lines which carry the genes for the efficient biosynthesis/accumulation of essential minerals, vitamins, and other health beneficial compounds. Conventional breeding involves crossing of best performing lines and selection of the lines with the targeted trait for many generations. However, the limited genetic diversity in exiting cultivars, unfavorable environmental interactions, and the time required for developing new cultivars are the key hurdles in conventional breeding. Thus, breeding can also be combined with mutagenesis or marker-assisted selection to reduce several limitations of conventional breeding. Genetic engineering on the other hand involves the introduction of the target trait as genetically modified DNA and the selection of best performing plants in a single

generation. However, genetic approaches must be combined with appropriate agronomy in order to bring out the full potential of the newly developed biofortified plant.

The first step in process of genetic biofortification is to identify suitable parents for the crossing program. Parents are identified after screening of the vast germplasm available for the cereal crops and their wild relatives. For iron and zinc biofortification in cereal grains, this task is somewhat complex because the grain concentration of these minerals depends on many physiological factors. When enough genetic variation is available, the breeders mainly rely on transgressive segregation, additive genetic effects, and heterosis for improving the targeted traits. Gyawali et al. (2019) reported higher correlations among Zn, P, and Mg, in addition to significant ($p < 0.01$) positive correlations between S-Fe, S-Zn, S-Cu, S-Mg, S-Mn, and S-P, thereby possibly indicating correlated and/or the common pathways of element uptake and translocation of these elements into the barley grains. The positive correlations among Zn, P, and Mg might be due to the well-known phytate on the binding of Zn and Mg in the grain (Marschner 1995). These correlations are important due to the crucial role of sulfur containing amino acids as promoters enhancing bioavailability of these elements (Welch and Graham 2004). Further, the positive correlation of S with Fe, Zn, Cu, Mg, Mn, and P is also important in barley (Cakmak et al. 2010), wheat (Morgounov et al. 2007; Pandey et al. 2016) and lentil (Karakoy et al. 2012) due to the involvement of sulfur-based methionine on phytosiderophore production and enhancing Fe and Zn mobilization in the soil, uptake into the plants and translocation into the grains. These correlations suggest that a strategic selection index may be implemented to achieve a balanced biofortification of nutrients/elements in barley breeding programs in future.

The QTLs (quantitative trait loci) have helped in the better understanding of complex traits. Through conventional breeding, these multigenic traits were earlier difficult to improve. Many QTLs have been identified for iron and zinc, but most of these are not stable across locations. In cereal grains, QTL mapping has also demonstrated the role of epistasis in the expression of these traits through interactions with other loci. QTL for *GPC* (grain protein content) has been mapped. This locus has positive effect on the high iron and zinc content in the grain. The *Gpc-B1* gene codes for NAM1, a NAC (NAM, ATAF, and CUC) transcription factor which belongs to "No Apical Meristem" (NAM) group of proteins in *Arabidopsis thaliana*. RILs and double haploid (DH) populations have been used for the mapping of iron and zinc QTLs. QTLs for both these minerals are found to be co-localized. Thus, iron and zinc biofortification in cereals can be accelerated by the identifying and tagging DNA markers related to these traits (Garcia-Oliveira et al. 2018).

Identification of genes or even QTLs responsible for phenolic metabolism is necessary for the genetic improvement of the trait. Although multiple studies have identified QTLs associated with phenolic compounds in rice and sorghum, there were few studies on total phenolic content, total flavonoid content, and antioxidant activity in barley. A genome-wide association study (GWAS) was conducted for total phenolic content, total flavonoid content, and antioxidant activity in 67 cultivated and 156 Tibetan wild barley genotypes. Most markers associated with phenolic content were different in cultivated and wild barleys. GWAS is an

efficient tool for exploring the genetic architecture of phenolic compounds. The DArT markers can be used in barley breeding for developing new barley cultivars with higher phenolics content. The candidate gene (HvUGT) provides a potential route for deep understanding of the molecular mechanism of flavonoid synthesis. These findings may serve as the foundation for further in-depth studies on molecular mechanism of natural variation in phenolic compounds.

GPC locus in barley (*HvNAM-1*) is a homologous gene of *GPC-B1 (TtNAM-B1)* from wild emmer wheat (*Triticum dicoccoides*) that controls leaf senescence and also results in N remobilization (Distelfeld et al. 2008). The remobilization of N compounds might improve translocation of Zn and Fe into seeds. The *GPC* locus might bring potential effects on micro-elements in barley, and its positive effect on Fe has been proved a study on a RIL population segregating for the SSR marker *Hvm74,* which is genetically linked to the *GPC* locus (*HvNAM-1*). A remarkable high genotype x environment interaction (GEI) is reported in phytate, phenolics, flavonoid, and Pi but relatively low GEI in Zn and Fe content suggesting strong genetic influences from *GPC* locus (Xue et al. 2016). In the phloem, zinc moves by binding to nicotianamine and other amino acids. By positional cloning of *Gpc-B1* in wheat, a NAC gene controlling concentrations of grain iron, zinc, and protein by remobilization of N from vegetative tissues is identified. In both wheat and barley, nutrient remobilization, protein content, and grain yield are considered to be regulated by leaf senescence. In barley, 48 NAC genes (*HvNACs*) including *Gpc-B1* ortholog genes have been identified (Hussain et al. 2016).

Zinc remobilization is an acritical factor that controls the accumulation of Zn irrespective of the soil zinc content. Limited phloem mobility of Zn is a major physiological barrier for the loading of zinc into grains. Genetic and molecular breeding approaches can be utilized to minimize this barrier. QTLs which regulate plant biomass, time to anthesis, the concentration of zinc in vegetative tissues, and Zn remobilization into grains have been identified with the help of genetic linkage maps in a double-haploid mapping population (Clipper X Sahara). The lines showed significant variation in grain zinc concentration (27–75 μg Zn/g) which correlated well with the zinc content remobilized into grains. These QTLs can be evaluated for trafficking of Zn into the grains and can be used in marker-assisted selection in zinc biofortification programs (Hussain et al. 2016).

Transgenic approaches aim at enhancing the availability of micronutrients to the plant from soil and rhizosphere; translocation to stem and leave and finally accumulation in the grains. With the availability of genome sequences, research into mineral biofortification has accelerated enabling forward and reverse genetics approaches. Transgenic studies utilizing iron homeostasis genes from model plants and their altered expression in cereal crops have proved useful in biofortification (Connorton and Balk 2019). The identification of genes underlying different QTLs directly using GWAS (genome wide association studies) has become cheaper and less time-consuming. QTLs have been mapped for mineral element content using 336 spring barley genotypes through GWAS by utilizing 6519 SNP markers (Gyawali et al. 2017).

Table 5.8 Transporters and chelators involved in the iron transport

Transporters	Chelators
YSL—Yellow stripe 1-like, a subfamily of the oligopeptides transporter (OPT) superfamily	Nicotianamine
ZIP—Zinc/iron-regulated transporter protein family	Mugineic acid
NRAMP—Natural resistance associated macrophage protein family	2′-Deoxymugineic acid
COPT—Copper transporter family	Hydroxylated derivatives of nicotinamide
CCC1—The Ca^{2+}-sensitive cross complementer1 family, also known VIT1 (vacuolar iron transporter) family	–
IREG—Iron regulated protein family	–

Transport of iron from vegetative tissues into the grain is very complex and involves a wide range of transporters and chelating agents (Table 5.8). Fe is transported mainly as Fe^{3+} ions bound to ITP (iron transport proteins). But, the Fe^{2+} ions are bound to nicotianamine and other mugineic acids inside the phloem. Uptake of Fe from soil can be improved by using the two strategies. Strategy I involves overexpression of genes for Fe (III) reductase genes and Fe^{2+} transporters of root plasma membrane. In strategy II, synthesis and exudation of phytosiderophores is increased along with increased expression of YSL protein gene.

The accumulation of iron can be improved by increasing the iron-sequestering capacity of edible tissues. This can be achieved through feedback mechanisms which influence the iron homeostasis in plant. Thus, the Fe content in the endosperm can be increased by altering the activity of vacuolar transporters like NRAMPs and VIT1 in grains (Borg et al. 2009). In plants, sequence similarity between the VITs is very high but differ in their biological functions. Thus, VITs are potential candidates for Fe biofortification because of their role in iron storage. *TaVIT2* is overexpressed in the wheat and barley endosperm, which resulted in more than two-fold increase in Fe content in white flour fractions without any significant effect on plant growth and grain number. Thus, bypassing the existing homeostasis mechanisms, more iron can be pumped into vacuoles in the endosperm, can be a successful biofortification strategy (Connorton et al. 2017).

Zinc in cereal grains is mainly found in the aleurone, pericarp, testa, and embryo portions with very low concentration in the endosperm. In barley and rice, Zn is also found in the subaleurone layers including outer endosperm. Therefore, enhancing the Zn accumulation in the endosperm is critical for human health as the outer layers are normally removed during milling/pearling. Constitutive overexpression of the plasma membrane Zn transporters *AtZIP1* and *HvZIP7* in barley has increased the grain Zn concentrations by 60% and 35%, respectively. Zinc content in endosperm can also be increased through expression of membrane transport proteins. *HvMTP1* transporter in barley is expressed mainly in phloem and aleurone cells, while its expression is low in the transfer and endosperm cells. Expression of *HvMTP1* under the control of the endosperm specific D-hordein promoter leads to the higher grain

zinc concentration in transformed plants as compared to controls without any significant change in the plant growth. But this transporter does not influence the Fe concentration in the grain. In the transformed plants, enhanced accumulation of zinc in the endosperm observed by the staining of grain cross-sections and also the redistribution of Zn takes place from the aleurone to the endosperm (Menguer et al. 2018). The barley plasma membrane P-type ATPase Zn transporter, *HvHMA2* is also an efficient candidate for mineral biofortification of crops. Transgenic homozygous barley lines overexpressing *HvHMA2* in the transfer cells of the grain results in doubling of a wide range of nutrients including zinc, iron, and magnesium in inner endosperm. Thus, the development of novel plants with enhanced zinc accumulation in endosperm can provide new opportunities to explore the bottlenecks limiting the grain zinc biofortification.

In addition to zinc, ZIP transporters can also transport a wide range of cations including iron. Transgenic barley plants expressing *Arabidopsis AtZIP1* are developed. Although the seeds of these plants had higher iron and zinc content as compared to control plants, they were wrinkled and small (Ramesh et al. 2004). It is suggested that modified CAX (calcium/proton exchanger) transporters can also be utilized to enhance zinc concentration in grains.

Concentration of the Se in the grains depends upon the ability of the plant to take up Se from the soil, to distribute it to different vegetative tissues, and to accumulate finally in the edible tissues in nontoxic forms. Se/S transporters in the root cell plasma membrane play important role in the uptake of Se by the plants. Adequate allelic variation in the domains which confer Se/S selectivity in HASTs (high-affinity sulfate transporter), in combination with the constitutive expression of Se-selective HASTs, should be utilized to increase Se concentrations and tissue Se/S quotients. The bioavailability of dietary Se not only depends on the amounts but also on the chemical forms of Se. In human diets, the line dividing the selenium deficiency and toxicity is very thin. Se in the form of SeMet (selenomethionine) and SeCys (selenocysteine) has greater bioavailability and can improve status of selenium in the vulnerable populations (White and Broadley 2009).

Simply increasing the level of different micronutrients in the cereal crops will not serve the purpose of biofortification. Along with the increased micronutrient concentrations, their bioavailability should also be increased in order to provide health benefits to the affected populations. The bioavailability of minerals can be improved either by decreasing the content of anti-nutritional factors like phytate, oxalate, and phenolic compounds or by enhancing the content of like β-carotene, ascorbate, and cysteine rich peptides etc. in the cereal grains. Different transgenic approaches can be utilized to reduce phytate content in the grain. Phytate content has been reduced in few crops by reducing the expression of genes involved in synthesis or sequestration of IP6. Overexpression of phytase genes have also led to the reduced phytate content in seeds. In barley, expression of phytase gene (*HvPAPhy*) in the grains has increased the bioavailability of iron and zinc. Vitamin E is a natural antioxidant present in the cereal grains. Its activity can be enhanced by co-expression of *At-VTE3* and *At-VTE4* genes (2-methyl-6-phytylbenzoquinolmethyltransferase), which increases δ-tocopherol and decreases γ-tocopherol content (Garg et al. 2018).

Root architecture and size play important role in determining access to soil nutrients and water. Rhizosphere chemistry can be modified by root architecture so that pH of the soil can be altered by an increase in secretion of root exudates, which can increase acquisition of minerals in the plant roots. Growth and branching of roots are reported to be negatively regulated by cytokinin hormone. In barley, root-specific expression of a cytokinin-degrading *CKX* gene leads to the formation of a larger root system which in turn results in higher micronutrient concentrations in shoot organs. Seeds of the transformed plants contained up to 44% more Zn. This trait has also been tested in field trial. This is an interesting finding, but the underlying mechanism is as yet unknown. Zn enrichment through root enhancement can be a low-cost sustainable strategy for genetic biofortification, which could be used in combination with other efforts (Ramireddy et al. 2018a, b).

Many barley genes involved in higher uptake of iron and zinc have been utilized for biofortifying other cereal crops which lack these genes. The overexpression of *HvIDS3* and *HvNAS* genes in rice grains has resulted in enhanced accumulation of iron and zinc in the grains. Mugineic acid production is increased in rice plants by the expression of barley *HvNAS1* (nicotianamine synthase) gene, two nicotianamine aminotransferase genes (*HvNAAT-A* and *-B*), and a mugineic acid synthase gene (*IDS3*) along with *ferritin* gene (*SoyferH2*) under the control of endosperm specific promoters. In these transgenic plants, Fe accumulation increased by 2.5–4.0 fold in the polished grains (Shahzad et al. 2014).

5.4 Agronomic Biofortification

Agronomic biofortification involves the application of the micronutrients either to the soil or as a foliar spray to enhance the content of the micronutrients in the grains. This is a quick and effective method to enhance concentrations of micronutrients in edible parts of the crop. Although it gives immediate results, but in the long run, genetic biofortification may be more cost-effective. Micronutrient fertilization is most effective when combined with NPK, organic fertilizers and high-yielding popular crop varieties. Thus, integrated soil fertility management practices should be followed to get best results from agronomic biofortification strategies. Agronomic interventions have been successfully used in many crops including barley. Significant increase in the nutritional quality and yields is reported, but the direct evidences indicating improved human health from these interventions are still lacking. The soils which are under cereal cultivation in the world are highly deficient in Zn. The pH of the soil plays very important role in the Zn availability to the plant roots. Higher soil pH, low organic matter and soil moisture content reduces the Zn availability drastically. Thus, the main reason of widespread Zn deficiency in crop plants is the low solubility of Zn in soils rather than low Zn content in the soil. Hence, under such soil conditions, the genetic potential of the newly developed biofortified varieties to absorb sufficient Zn from the soil and to accumulate it in the grain in order to achieve the targeted nutritional benefit may not be expressed to the full extent. Thus, agronomic biofortification either by application of Zn fertilizers to

the soil and/or by foliar application seems to be very promising to ensure success of breeding efforts for enhancing Zn concentration in cereal grains.

In cereal grains, there are two main sources of Zn: (1) continuous uptake of Zn from the soil and its translocation into the grains and (2) Zn deposited in vegetative tissues is remobilized to the grains during the reproductive stage. Many soil and plant factors influence the relative contributions of these two sources to accumulation of Zn in grains. These factors include the availability of micronutrients and water during grain filling period, length of the grain filling period, nitrogen status of the soil/plant, and the timing of senescence. Therefore, maintaining the adequate amount of readily available Zn in the soil and in the vegetative tissues during grain filling is utmost important for the Zn biofortification of the cereals.

Zinc sulfate and EDTA-chelated Zn are the commonly used salts for the foliar application. Zn-EDTA is comparatively more effective than zinc sulfate in enhancing the grain Zn concentration after foliar spray. But Zn-EDTA is highly priced than zinc sulfate. Thus, zinc sulfate can be a cost-effective option for agronomic Zn biofortification. The time of foliar application of Zn fertilizer is a very critical factor in determining the effectiveness of the biofortification program. The foliar application of Zn is more effective in the later stages of plant development particularly after flowering and especially during the grain filling stage. The foliar applied Zn is phloem-mobile and can be easily translocated into developing grains. Localization and speciation studies in cereal grains indicate that Zn interacts with proteins and the grain proteins constitute a physiological sink for Zn (Cakmak and Kutman 2018). Many research studies in Turkey have showed that the application of Zn fertilizers to cereal crops like wheat, maize, barley, and sorghum increased yields and grain Zn concentrations (Cakmak 2010). Mineral content in the crop plants can be regulated not only by the fertilization with micronutrients, but also with macronutrients like nitrogen, phosphorus, potassium, magnesium, and sulfur. Plant nitrogen status plays a significant role in Zn biofortification of cereal grain. Improving the nutritional status of nitrogen in plants increases Zn accumulation in grain. Soil application of nitrogen and zinc improves plant height, flag leaf area, dry matter production, and grain yield. In barley, application of NH_4NO_3 and $ZnSO_4{\cdot}7H_2O$ (10 mg/kg soil each) to the soil at pre-sowing and foliar application of 0.5% $ZnSO_4{\cdot}7H_2O$ solution at different plant growth stages significantly increased yield as well as the Zn concentrations in grains of barley. NH_4NO_3 maintains the pH of soil by acidifying effect which increases the availability of zinc to the treated plants as compared to control (Yadav and Sharma 2018).

Sulfur is a macronutrient and plays important physiological roles in plant metabolism. A progressive deficiency of this nutrient has been observed in soils of many regions of the world. Application of Sulfur fertilizers to the crops cultivated on deficient soils positively affects the content of grain micronutrients (zinc, iron, copper, and manganese). Fertilization of spring barley with NPK supplemented with Sulfur (40 kg S/ha of potassium and ammonium sulfates) enhances the absorption of micronutrients by spring barley. Thus, because of the positive effects on the level of various micronutrients, sulfur fertilization can be included in the barley biofortification programs for better results (Barczak et al. 2019).

Selenium is a very important mineral for both the humans and animals. The amount of selenium in a food sources is mainly determined by the quality of the soil. Other factors like rainfall, evaporation, and pH levels also affect selenium concentration in soil. Selenium deficiency is more common in certain parts of the world and an estimated 1 billion people around the world are affected by selenium deficiency (Jones et al. 2017). Se deficiency can lead to reduced metabolic functioning, decreased immunity, impeded growth, cognitive impairment finally leading to reduced productivity. Many evidences indicate that throughout the world, the intake of Se by most of the people is not adequate and that supplementation can provide beneficial effects. However, the bioavailability of inorganic Se is less than the organic form of Se. Therefore, the main aim of Se biofortification should be to convert Se from inorganic form to organic forms by incorporation into the proteins as selenomethionine. Agronomic biofortification can play important role in enhancing the selenium content in barley through fertilizer, foliar application or addition during malting process. Sodium selenate is mainly used for foliar application because of its weak adsorption on soil colloids. It can lead to rapid increase in plant Se levels without affecting crop yield. Sodium selenate application immediately after anthesis on the plants and its addition during the germination stage of the malting process results in enhanced accumulation of Se in barley grains. Also, the final beer products contain a substantial amount of Se (Gibson et al. 2006). In two-row barley, for each gram of sodium selenate applied, 44 μg/kg dry weight increase in total Se in the grain is reported. Even after the highest dose of fertilizer, no increase in the total Se level is observed in the soil. More efforts are required to enhance the Se content of barley for both food and fodder purpose and agronomic interventions can play an important role (Rodrigo et al. 2013).

Unlike Zn and Se, the application of iron-enriched fertilizers to the soil is not effective in increasing the mineral content of the grain. This is because, in the soil, Fe precipitates to insoluble forms which are not available for absorption by plants. Thus, for Fe enrichment, the most effective agronomic practice could be through foliar application of mineral iron. Contrasting results are reported in literature where, some studies have reported increase in Fe concentrations after foliar applications, while others reported no response of foliar application.

6 Bioavailability of Nutrients

The bioavailability of nutrients is a better indicator of nutritional quality of a food crop than the bioaccumulation of nutrients. The type of food matrix is an important factor determining the bioavailability of organic and inorganic compounds. The presence of enhancers and inhibitors in the food also determine the bioavailability of the mineral elements. Only 5% of the iron and 25% of the zinc are reported to be bioavailable in cereal and legume seeds (Pfeiffer and McClafferty 2007). The dietary phytate/Fe and phytate/Zn molar quotients play important role in determining the bioavailability of iron and zinc. A phytate/iron molar quotient greater than one

reduces the bioavailability of iron and a phytate/Zn molar quotient greater than six reduces the bioavailability of zinc (Lönnerdal 2002).

Therefore, the bioavailability of nutrients can be enhanced by reducing the levels of anti-nutritional factors or increasing the levels of nutritional enhancers. The chemical form of the mineral nutrient also determines the bioavailability to a greater extent. In case of selenium, an organic form of Se such as selenomethionine is more efficiently absorbed than the inorganic metal ions. Likewise, in comparison to non-heme iron, Fe-ferritin complex is less affected by the anti-nutritional compounds (Zhu et al. 2013). Food processing like milling/pearling and the speciation of the micronutrients within the grain are also the important factors which decide the bioavailability of micronutrients for absorption by the human gut (Detterbeck et al. 2016). Therefore, it is very important to generate detailed information on the localization of micronutrients within the grain and whether any variation in this respect exits between genotypes with contrasting micronutrient concentrations. High micronutrient concentrations have been reported in the embryo followed by husk and then the endosperm (Detterbeck et al. 2016).

6.1 Enhancers

Enhancers are the substances in the grains that enhance mineral nutrients bioavailability. Some promoters also decrease the activity of inhibitors. Other promoters influence the accumulation of mineral nutrients in grains with foliar application. Silicon improves crop production by increasing water uptake, promoting photosynthetic rate, maintaining nutrient balance, and increasing the activities of antioxidants. Hormones like brassinosteroids increase tolerance to stress and mineral nutrients uptake. Chitosan chelates minerals and other nutrients and is used widely in phytoremediation for heavy metal removal. Foliar application of chitosan in barley is also found effective in increasing the level of promoters like β-carotene and glutathione and reducing of Phy/yellow pigment ratio which finally results in the increased bioavailability of Ca, Mg, Fe, Zn, and Mn (Dragičević et al. 2016).

Phytic acid acts as the major storage form of phosphorus in plants and leads to decreased bio-availability of micro-nutrients. The genotypes with lower phytic acid content are desirable with respect to increased availability of nutrients. Low phytic acid mutants of barley have been developed by chemical mutagenesis. The lpa mutations show drastic reductions in phytic acid P to inorganic P ratio. The lpa mutations result in different biochemical phenotypes in barley. The most common type of mutation, lpa1 shows decrease in phytic acid P with a molar-equivalent increase in inorganic P. While the lpa2 mutation shows decrease in phytic acid P without any molar-equivalent increase in inorganic P. Instead, in the lpa2 mutants, a major part of the total seed P remains bound to the lower inositol polyphosphates, e.g., myo-inositol pentakisphosphate (IP5). All lpa phenotypes are due to single-gene, recessive mutations which result in several fold increases in inorganic P (5–10-fold for lpa1 and 3–4-fold for lpa2) and significant decrease in phytic acid P (50% or more) in comparison to normal seeds. These lpa mutations can be successfully used

in biofortification programs to reduce the phytic acid content and increase the bioavailability of mineral elements (Larson et al. 1998).

Another way to increase the bioavailability of micro-nutrients can be by developing genotypes with higher phytase activity. The enzyme phytase (*myo*-inositol hexakisphosphate phosphorylase, EC 3.1.3.8) hydrolyses phytic acid to myo-inositol and inorganic phosphate. Significant positive correlations are reported between native phytase activity and phosphorus utilization and micronutrient bioavailability. Thus, phytases can play crucial role in reducing the phytic acid content in barley products thereby enhancing the bioavailability of many essential micronutrients from cereal based diets in humans. In this direction, QTLs controlling phytase activity have been identified in barley doubled haploid population. In barley, a gene controlling phytase activity (HvPAPa) has been mapped to chromosome 5H. The phytase enzyme in barley is identified as purple acid phosphatase (PAP). The understanding of phytase genetics in barley can provide effective tool for breeding barley with higher phytase activity (Dai et al. 2011).

6.2 Enhancement of Bioavailability with Processing

In resource-poor populations, malnutrition is not only due to unavailability of sufficient quantity of food but is also due to the low content of essential micronutrients in the staple diets. Even if the food with adequate level of micronutrients is available, the bioavailability of these nutrients is mostly low from the staple foods because of the presence of many anti-nutritional factors. To overcome such limitations, different strategies appropriate for the rural poor have to be considered and implemented. Many traditional household food preparation and processing and methods can be used to improve the bioavailability of micronutrients in staple plant-based foods. These methods include soaking, germination/malting, fermentation, and mechanical/thermal processing. These techniques reduce the content of anti-nutrients and enhance the physico-chemical accessibility of several micronutrients. In addition to the traditional processes, new processing methodologies are now available for producing value added food products with enhanced nutritional properties and without losing their microbiological safety.

Suitable processing technologies can enhance the bioavailability of the bound phenolic compounds. This can be achieved primarily through particle size reduction, structural breakdown of cereal matrices and their release from cereal matrices. Extrusion cooking and thermal treatments of cereal grains may affect bioavailability of phenolic compounds either positively or negatively as high temperatures may cause decomposition of heat-labile phenolic compounds or result in polymerization of some compounds during high-pressure extrusion cooking. In cereal grains, the bioavailability of phenolic compounds depends on the grain type and the processing method, and the conditions used. The mechanical processing and bio-processing have positive effects on the bioavailability of grain phenolic compounds. Thus, use of a proper combination of these two processing methods is worth investigating in future. The adverse effects of including bran fractions in food formulations can be

reduced by using properly processed bran and whole grains. This is an important research area which needs further investigation to improve the nutritional quality of food products (Wang et al. 2014).

7 Future Prospects

Barley is a very important staple crop in several parts of the world with widest range of cultivation from cold deserts/higher mountain elevations to the sea levels and often considered as the last crop before hot Sahara deserts. It has lesser inputs requirement also as compared to other cereals and thus can be grown with relatively lesser resources vis-a-vis other cereals. But barley grows well in irrigated areas or areas with moderate rainfall also. Barley matures earlier and has evolved to use less heat units than other crops. Therefore, barley is currently being referred as "crop for climate change" for its capacity to adapt with wider climatic conditions from extreme cold to hot environments as well as huge cropping days differences, in addition to its capacity to perform under low input conditions. Enhancing the nutritional value of barley is very important in order to reduce the malnutrition. According to the proposed CGIAR 5-year biofortification strategy 2019–2023, the high β-glucan content trait in barley will be recombined with high Fe and Zn controlling genes currently available within ICARDA germplasm. The high β-Glucan, Fe and Zn containing advanced lines generated through double haploid and conventional RIL mapping population will be made available to plant breeders from developing country which will contribute to nutritional security in rural-urban communities across the globe. By the end of the project high β-glucan, Zn and Fe containing hull less barley will be produced and food barley with superior bread making quality will be available to test and integrate into food barley breeding programs, and for mass scale production in the farmers' field.

In future, there is need to develop more efficient methods of foliar application of Zn in barley to enhance uptake of Zn by the plants and its accumulation in the grains. If the bioavailability of Zn is higher after foliar spray than the soil application, this strategy would be very effective in solving Zn related health problems throughout the world. There is need to develop barley lines with high phytase activity. For this new phytases need to be discovered and engineered. Further research is required to optimize the dose and delivery of phytase into the human foods.

The impact of various food processing techniques used at household level to enhance the nutritional quality of food products should be assessed in well-designed efficacy trials. This is especially needed for the reduction of phytate using long-term feeding trials in order to assess the measurable impact on the mineral bioavailability. Suitable strategies can be integrated with interventions that in particular provide health and nutrition education at community level. Adoption of the newly developed biofortified varieties by the farmers would be very challenging. Farmers will grow such crops only if no addition inputs will be required, yield is at par and most importantly they get a premium price for their produce. Moreover, the consumers

will accept the food and food products from the biofortified crops only if they are not expensive than the normal food items and secondly there are no appreciable changes in the color, texture, taste, and cooking/baking quality of the food products. There will be a need of awareness programs at community level which will demonstrate the health benefits of the biofortified foods and influence the choice of the consumers.

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Biofortification of Maize for Nutritional Security

6

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Abstract

Nutritious diet is vital for proper growth and development in humans. It helps in preventing diseases, besides maintaining the body metabolism for physical and mental wellbeing. Consumption of unbalanced diet leads to malnutrition which affects most of the world's population from infancy to old age. It affects all geographies, age groups and people from rich to poor. Dried grains from the field maize serve as an important source of both food and feed. Besides, specialty maize such as sweet corn, waxy corn and popcorn have become popular choices, and generated livelihood worldwide. However, available maize is deficient in essential amino acids (lysine and tryptophan), vitamins (vitamin-A and vitamin-E) and minerals (iron and zinc). People dependent on maize-based diet develop symptoms that affects their health and work efficiency leading to the serious socio-economic implications. Availability of natural mutants of key genes has provided a great opportunity to develop nutrient-rich maize through breeding approaches. Associated markers for these genes help in developing nutritious maize cultivars through molecular breeding. Transgenic approach also provides newer avenues to develop nutrition-rich maize genotypes. Here, we have presented the status of research and development on biofortified maize cultivars for various nutritional traits. Impact of biofortified maize on human health, and growth and development of chickens and pigs has been mentioned. Various aspects of challenges for higher adoption and popularization have also been discussed.

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1 Introduction

Malnutrition caused due to consumption of unbalanced food has impacted all strata of the population worldwide (Hossain et al. 2019a; Prasanna et al. 2020). Two billion people are the victims of malnutrition, of which 820 million are undernourished. The poor nations recorded 10-times more undernourished people compared to the richer nations, while obesity—another form of malnutrition—was five-times higher in the rich nations than in poor nations (Global Nutrition Report 2020). The sustainable development goals (SDGs) set by the United Nations aim to eliminate malnutrition in all forms by 2030. Nutrients such as amino acids, vitamins and minerals are essential and required in low amount, but they play a vital role in stimulating cell development, signalling, functioning and metabolism in humans (Gupta et al. 2019; Hossain et al. 2019b). Since the human body cannot synthesize the majority of the nutrients, one has to rely on balanced diet (Fitzpatrick et al. 2012). Even in the present day, COVID-19 has an instant direct effect on food systems in developing countries, as large proportion of people are at higher risk due to prevailing micronutrient deficiencies and poor nutritional status of the body (McAuliffe et al. 2020). Global population depends on cereals such as wheat, rice and maize, which are often low in key essential nutrients and way below the level to meet the recommended daily allowances (RDA) (Jha and Warkentin 2020).

Maize is one of the three topmost cereals being cultivated and consumed across the globe (Shiferaw et al. 2011). The projected maize production for 2020 is around 1197.77 million metric tonnes with cultivation of 200.30 million hectares area (FAS-USDA 2021). Maize and its products account 65% of Africa's food supply, 30% in America and 6.5% in Asia, justifying its significance in food security and economic development (Prasanna et al. 2020). The various special types of maize called 'specialty maize' viz., sweet corn, waxy corn, popcorn, and baby corn have become popular and consumed across the countries by people of different ages. Maize provides the significant number of calories in the daily diet; however, it is deficient in essential amino acids, micronutrients and vitamins (Hossain et al. 2019a; Gupta et al. 2019; Prasanna et al. 2020). Maize contains ~10% of protein, but poor in its quality and thereby excessive consumption of corn deficient in essential amino acids results in increased susceptibility to diseases and protein-energy malnutrition (Bain et al. 2013; Gupta et al. 2015). Maize is also deficient in provitamin-A with very low level of 1–2 μg/g (Vignesh et al. 2012; Muthusamy et al. 2015a, b; Zunjare et al. 2017). In maize, among various isoforms (α, β, δ, γ) of tocopherol, α-tocopherol possesses the highest vitamin-E activity, but constitutes only ~20% of the total tocopherol leading to the deficiency of vitamin-E (Egesel et al. 2003; Das et al. 2020). Iron (Fe) and zinc (Zn) that are required for vital functions in the body are also deficient in maize grains (Jaiswal et al. 2019; Abhijith et al. 2020).

Therefore, it is necessary to enhance the level of essential nutrients in maize endosperm to alleviate malnutrition especially among the population dependent on maize as staple (Tanumihardjo 2011).

Biofortification is the process of developing nutrient-rich crops through breeding approaches, and it provides sustainable and cost-effective solution to address malnutrition (Yadava et al. 2018). 'Food-fortification', 'dietary diversification' and 'medical-supplementation' strategies often used as a tool to provide better nutrition, possess limited success in resource-limited environments and poor countries where healthcare and food-processing facilities are not well organized (Pfeiffer and McClafferty 2007; Neeraja et al. 2017). The sustainable and genetic improvement method also known as 'crop biofortification' of staple crops for the target micronutrient provides a viable alternative approach as it delivers the products enriched with target micronutrients in natural form (Tako et al. 2013). The strategy of biofortification is now being followed by many countries to enrich the staple food crops with micronutrients. In India, series of biofortified varieties were developed and subsequently released and notified for commercial cultivation (Yadava et al. 2018).

Marker-assisted selection (MAS) has significantly improved the efficiency of breeding through providing benefits such as reduction in time, elimination of large-scale phenotyping, selection at seedling stage, avoiding linkage drag, combining multiple genes simultaneously, and feasibility of improving traits of low heritability. MAS is now being widely used to accelerate the development of biofortified varieties through selection/introgression of major gene(s)/quantitative trait loci (QTL) (Prasanna et al. 2020). In maize, favourable alleles of genes viz., *opaque2* (*o2*) (Mertz et al. 1964), *opaque16* (*o16*) (Yang et al. 2005) for essential amino acids, *β-carotene hydroxylase* (*crtRB1*) (Yan et al. 2010) and *lycopene epsilon cyclase* (*lcyE*) (Harjes et al. 2008) for provitamin-A (proA), *γ-tocopherol methyl transferase* (*vte4*) (Liu et al. 2012) for provitamin-E (proE), *lpa1-1* and *lpa2-1* (Raboy et al. 2000) for low phytic acid have been identified, and markers are available in the public domain for use in molecular breeding (Table 6.1). Though the majority of efforts were targeted towards nutritional enhancement of field corn types, few research efforts were also attempted towards biofortifying sweet corn, popcorn and waxy corn in different countries (Table 6.1). We present here the status of nutritional quality enhancement in maize, their impacts on human health, and various challenges in the popularization of the biofortified maize technologies.

2 Nutritional Enhancement of Field Corn

2.1 Improvement for Quality Protein

Maize deficient in essential amino acids viz., lysine and tryptophan contribute to various protein-related health problems and in extreme cases leads to protein-energy malnutrition (PEM) (Vasal et al. 1980). '*Kwashiorkor*' and '*Marasmus*' are the two well-known diseases developed due to insufficient intake of protein (Bain et al.

Table 6.1 List of MAS programmes undertaken for nutritional enrichment in maize

S. no.	Trait(s) improved	Gene(s) introgressed	Marker(s) used	Reference(s)
1.	Lysine	*o16*	*umc1141*	Zhang et al. (2010)
2.	Lysine	*o16*	*umc1141*	Yang et al. (2005)
3.	Tryptophan	*o2*	*phi057*	Jompuk et al. (2011)
4.	Provitamin-A	*crtRB1*	*3′TE InDel*	Natesan et al. (2020)
5.	Lysine and tryptophan	*o2*	*phi057*	Jompuk et al. (2020)
6.	Lysine and tryptophan	*o2*	*phi057*	Shetti et al. (2020)
7.	Lysine and tryptophan	*o2*	*umc1066*	Pukalenthy et al. (2020)
8.	Lysine and tryptophan	*o2*	*umc1066*	Pukalenthy et al. (2019)
9.	Lysine and tryptophan	*o2*	*umc1066* and *phi057*	Chand et al. (2019)
		o16	*umc1149* and *umc1141*	
10.	Lysine and tryptophan	*o2*	*umc1066* and *phi057*	Hossain et al. (2018)
11.	Lysine and tryptophan	*o2*	*umc1066*	Ren et al. (2018)
12.	Lysine and tryptophan	*o2*	*phi112, phi057* and *umc1066*	Sarika et al. (2018b)
		o16	*umc1141* and *umc1149*	
13.	Lysine and tryptophan	*o16*	*umc1149*	Sarika et al. (2017)
14.	Lysine and tryptophan	*o2*	*umc1066*	Surender et al. (2017)
15.	Lysine and tryptophan	*o2*	*phi057*and *umc1066*	Kostadinovic et al. (2016)
16.	Lysine and tryptophan	*o2*	*phi057* and *phi112*	Zhou et al. (2016)
17.	Lysine and tryptophan	*o2*	*umc1066*	Gupta et al. (2013)
18.	Lysine and tryptophan	*o2*	*phi112*	Zhang et al. (2013)
		o16	*umc1121*	
19.	Lysine and tryptophan	*o2*	*phi057*	Magulama and Sales (2009)
20.	Lysine and tryptophan	*o2*	*phi057, umc106* and *phi112*	Danson et al. (2006)
21.	Lysine and tryptophan	*o2*	*phi057* and *phi112*	Manna et al. (2005)

(continued)

Table 6.1 (continued)

S. no.	Trait(s) improved	Gene(s) introgressed	Marker(s) used	Reference(s)
22.	Lysine and tryptophan	*o2*	*umc1066* and *phio57*	Babu et al. (2005)
23.	Lysine, tryptophan and provitamin-A	*crtRB1*	*3′TE-InDel*	Mehta et al. (2020a)
		o2	*umc1066*	
24.	Lysine, tryptophan and provitamin-A	*crtRB1*	*3′TE InDel*	Chandran et al. (2019)
		o2	*umc1066*	
25.	Lysine, tryptophan and provitamin-A	*crtRB1*	*3′TE InDel*	Goswami et al. (2019)
		o2	*phi057* and *umc1066*	
26.	Lysine, tryptophan and provitamin-A	*crtRB1*	*3′TE InDel*	Zunjare et al. (2018a)
		lcyE1	*5′TE InDel*	
		o2	*phi057*	
27.	Lysine, tryptophan and provitamin-A	*crtRB1*	*crtRB1–5′TE-2* and *crtRB1–3′TE-1*	Liu et al. (2015)
28.	Lysine, tryptophan and provitamin-A	*o2*	*umc1066*	Muthusamy et al. (2014)
		crtRB1	*3′TE InDel*	
29.	Provitamin-A	*lcyE*	*lcyE5′TE* and *3′InDel*	Yang et al. (2018)
30.	Waxy, lysine and tryptophan	*wx1*	*phi027*	Wang et al. (2019)
		o2	*umc1066*	
31.	Waxy, lysine and tryptophan	*wx1*	*phi022, phi027* and *wx-2507F/RG*	Talukder et al. (2019)
		o2	*phi057*	
32.	Waxy, lysine and tryptophan	*wx1*	*phi027*	Zhou et al. (2016)
		o2	*phi057*	
33.	Waxy, lysine and tryptophan	*wx1*	*phi022, phi027* and *phi061*	Yang et al. (2013)
		o16	*umc1141*	
34.	Waxy, lysine and tryptophan	*wx1*	*phi027*	Zhang et al. (2013)
		o16	*umc1121*	
		o2	*phi112*	
35.	Waxy, lysine, tryptophan and provitamin-A	*wx1*	*phi022, phi027* and *wx-2507F/RG*	Mishra et al. (2019)
		o2	*phi057*	
		crtRB1	*3′TE InDel*	
36.	Vitamin-E	*vte4*	*InDel7* and *InDel118*	Xiao et al. (2020)
37.	Vitamin-E	*vte4*	*InDel7* and *InDel118*	Feng et al. (2015)
38.	Low phytate	*lpa2-2*	*umc2230*	Sureshkumar et al. (2014)
39.	Low phytate	*lpa2-2*	*umc2230*	Tamilkumar et al. (2014)
40.	Anthocyanin	*Pr1*	*nc009*	Lago et al. (2013)
		B1	*umc1776*	

2013). PEM among all nutritional disorders has been the leading cause of highest number of deaths worldwide. Daily requirement of lysine is 30 mg/kg body weight for adults and 35 mg/kg body weight for children of 3–10 years of age. Tryptophan is required at the rate of 4 mg/kg body weight for adults and 4.8 mg/kg body weight in children in a day (WHO/FAO/UN 2007). It is estimated that an additional 122 million people could experience PEM at the end of 2050 (Grebmer et al. 2019).

The *o2* mutant was discovered by Jones and Singleton in the 1920 at Connecticut maize field in the USA (Vivek et al. 2008). This naturally available mutant *o2* allele located on chromosome 7 increases lysine and tryptophan by almost 2-folds (Mertz et al. 1964). At molecular level, it codes a leucine zipper transcriptional factor that regulates the transcription of 19- and 22-kDa α-zein. Recessive *o2* genotypes have lesser synthesis of α-zein (deficient in lysine and tryptophan) and concurrent increase in non-zein (rich in lysine and tryptophan) resulting in enhancement of nutritional quality. Mutant *o2* also reduces the transcription of lysine keto-reductase that degrades lysine in maize endosperm (Kemper et al. 1999). However, *o2* was found to have several negative pleiotropic effects such as soft, starchy textured opaque grain which make the grains more susceptible to mechanical damage, storage pests and fungal diseases (Hossain et al. 2007a, b, 2008a). The chromosomal regions that affect the conversion of soft to hard kernel are called as 'endosperm modifier genes' (Vasal et al. 1980; Pandey et al. 2015). The pyramiding of these modifier loci in the *o2* genetic background led to the birth of hard endosperm-based nutritionally enriched maize, popularly called as quality protein maize (QPM) (Hossain et al. 2008b). Recently, a natural recessive *o16* mutant mapped on chromosome 8 has been associated with a higher concentration of lysine and tryptophan (Yang et al. 2005; Sarika et al. 2017, 2018a).

Several QPM cultivars have been developed through conventional breeding worldwide (Tandzi et al. 2017). Some of the very popular QPM cultivars are Obatanpa (Ghana), AMH760Q (Ethiopia), Longe-5 (Sudan), Poshilo Makai-1 (Nepal), Yunrui-1 (China) and Chaskarpa (Bhutan) adapted in tropical and subtropical environments have been developed (Vasal 2001). In Bangladesh, QPM hybrid, BARI Hybrid Maize-5 was released. Pakistan also released QPM hybrids, QPHM200 and QPHM300 during 2017. In India, several QPM hybrids viz., Shaktiman-1, Shaktiman-2, Shaktiman-3, Shaktiman-4, Shaktiman-5, HQPM-1, HQPM-4, HQPM-5, HQPM-7 and Pratap QPM Hybrid-1 have been released for commercial cultivation (Gupta et al. 2015). Gene-based SSRs (*phi057*, *phi112* and *umc1066*) specific to *o2* were used to convert popular hybrids into QPM version. 'Vivek QPM-9' (Gupta et al. 2013), 'Pusa HM4 improved', 'Pusa HM8 improved' and 'Pusa HM9 improved' have been successfully developed through molecular breeding in India (Hossain et al. 2018). Furthermore, gene linked SSR markers viz., *umc1141* and *umc1149* were utilized for introgression of *o16* and pyramiding with *o2* and *wx1* genetic background (Zhang et al. 2013). Nearly, half-fold increase in lysine among *o2o2/o16o16* progenies over *o2o2* progenies was reported in China (Yang et al. 2005; Zhang et al. 2010). In India, *o2* and *o16* genes were pyramided successfully in the genetic background of four QPM released hybrids viz., HQPM-1,

HQPM-4, HQPM-5 and HQPM-7 (Sarika et al. 2018b). Recently, Chand et al. (2019) have pyramided *o2* and *o16* into parental lines (HKI1344, HKI1378 and HKI1348-6-2) of popular white hybrids viz., HM5 and HM12 (Table 6.1).

Transgenics route has also been explored to enhance the lysine level in maize. Deletion of basic domain and first 175 N-terminal residues in wild type *O2* allele led to the creation of mutant *o2* allele (Unger et al. 1993). The ten-fold reduction in 22-kDa α-zein caused the increase in lysine and tryptophan due to concurrent enhancement of non-zein proteins. Further, RNAi constructs against 22- and 19-kDa α-zeins were developed, and transgenic plants showed a significant reduction in the synthesis of zeins and recorded high lysine concentration (Wu et al. 2010). Segal et al. (2003) also developed RNAi constructs derived from a 22-kDa α-zein, and produced a dominant opaque phenotype. However, transgenic maize high in lysine is yet to be deployed for commercial production.

2.2 Improvement of Provitamin-A

Vitamin-A deficiency (VAD) causes eye visibility issues like night blindness, keratomalacia, besides diarrhoea and respiratory diseases (Sommer and Davidson 2002; Mayer et al. 2008). VAD may also cause disorders like stunting in growth, impaired iron mobilization, lower immune response, and increased susceptibility to infectious diseases (WHO 2009). Nearly 4.4 million preschool-age children and 20 million pregnant women suffer due to vitamin-A deficiency globally (www.harvestplus.org). Among different proA carotenoids, α-carotene (AC), β-carotene (BC) and β-cryptoxanthin (BCX) serve as the precursors for vitamin-A biosynthesis, while lutein (LUT) and zeaxanthin (ZEA) act as scavengers for free radicals (Burt et al. 2010). The targeted amount for proA to meet the daily requirement in humans is 15 μg/g, as fixed by HarvestPlus, an international body for the development of nutrition-rich crops for better nutrition. However, traditional maize contains only 2–3 μg/g of proA (Pixley et al. 2013).

Favourable alleles of *crtRB1* and *lcyE* are the preferred choice by the plant breeders for the development of proA rich maize cultivars worldwide (Muthusamy et al. 2014, 2016; Andersson et al. 2017; Zunjare et al. 2018a; Prasanna et al. 2020). Availability of gene-based *InDel* markers has made molecular breeding an effective approach for proA enrichment (Harjes et al. 2008; Yan et al. 2010; Babu et al. 2013; Zunjare et al. 2018a). The mutant version of *crtRB1* gene located on chromosome 10 restricts the conversion of BC into BCX, and further BCX to ZEA, thereby enhances the accumulation of BC (Yan et al. 2010). *crtRB1* gene belongs to the fatty acid hydroxylase (FAH) superfamily which includes a broad spectrum of proteins involved in carotenoids hydroxylation and sterol desaturation in higher plants (Dutta et al. 2019). Another gene, *lcyE* located on chromosome 8, alters flux accumulation towards β-branch instead of α-branch of the pathway, and results in three-fold difference in proA carotenoids (Harjes et al. 2008). Furthermore, the pyramiding of both the genes in single genetic background enhances proA content greater than

either of the genes alone (Babu et al. 2013; Zunjare et al. 2017; Gebremeskel et al. 2017).

Several proA-rich hybrids were developed through breeding approach across the countries (Table 6.1) (Gupta et al. 2019). ProA-rich hybrids and synthetics released in countries like Zambia (GV662A, GV664A, GV665A), Nigeria (Ife maize hyb-3, Ife maize hyb-4, Sammaz 38, Sammaz 39) and Ghana (CSIR-CRI Honampa) are some of the important ones (Dhliwayo et al. 2014; Simpungwe et al. 2017). Molecular breeding for *crtRB1* and *lcyE* has led to the development and release of proA-rich hybrids viz., 'Pusa Vivek QPM9 Improved', 'Pusa Vivek Hybrid-27', 'Pusa HQPM-5 Improved' and 'Pusa HQPM-7 Improved' in India (Muthusamy et al. 2014; Zunjare et al. 2018b, c; Yadava et al. 2018). Use of genetic engineering is also another alternative approach for development of maize genotypes rich in proA carotenoids. For instance over expression of bacterial (*Erwinia herbicola*) genes such as *crtB* and *crtI* enhance BC content to a level of 10 μg/g in Hi-II maize line (Aluru et al. 2008). Zhu et al. (2008) and Naqvi et al. (2009) also developed transgenic maize genotypes with BC concentration as high as ~60 μg/g while stacking five genes together (*psy1*, *crtI*, *lycb*, *bch* and *crtW*). However, these transgenic-based proA-rich maize hybrids are yet to be deployed for commercial production.

The research attempts were also made to study the retention and post-harvest loss of proA carotenoids after a long period of storage (Taleon et al. 2017; Dutta et al. 2020a, b). On exposure of several physical factors such as light, heat and oxygen, there is a reduction in proA carotenoids under storage in maize grain (Boon et al. 2010; De Moura et al. 2015). The extent of loss in maize inbreds also vary depending upon different storage methods such as traditional, refrigerated and vacuum packing conditions (Dutta et al. 2020a). Oxidative reaction, including both enzymatic and non-enzymatic, is also responsible for the degradation of carotenoids (De Moura et al. 2015). Intriguingly, non-proA carotenoids (LUT and ZEA) have better retention than the proA compounds (BC and BCX) and majority of proA losses occur at the initial first 3 months of storage. Maize inbreds and hybrids with higher retention of proA during storage have been recently identified (Dutta et al. 2020a, b). Recently, *carotenoid cleavage dioxygenase 1* (*ccd1*) was identified which have wide range of activity on cleavage of carotenoid compound to generate the apocarotenoids (Vallabhaneni et al. 2010). It was found that high expression of *ccd1* gene leads to lower retention of carotenoids particularly for proA compounds (BC and BCX) at monthly intervals (Dutta et al. 2020c).

2.3 Improvement of Provitamin-E

Vitamin-E plays a vital role for scavenging of various reactive oxygen species and free radicals, quenching of singlet oxygen, and providing membrane stability, therefore essential in human growth and body metabolism (Muzhingi et al. 2017). Around 4 mg day/day for 0–6 month's old child, while the 15 mg/day for the adults is the recommended daily allowance for vitamin-E (Institute of Medicine 2000). The

deficiency of vitamin-E leads to age-related macular degeneration, neurological disorders, cancer, cataracts, Alzheimer's-, cardiovascular and inflammatory diseases (Bramley et al. 2000). It is estimated that 20% of the global population possesses sub-optimal level of vitamin-E (Li et al. 2012). In addition, vitamin-E (tocopherols) is also used in the pharmaceutical and cosmetics industry and also used as an animal feed additive to improve the quality and shelf-life of meat.

Vitamin-E is a group of amphiphilic molecules that includes tocopherols, tocotrienols and plastochromanol-8 (Vincent et al. 2020). Vitamin-E is made up of four isoforms (α, β, δ, γ), out of which, γ-tocopherol constitutes ~80% of the total tocopherol, while α-tocopherol is of ~20% of the total vitamin-E in maize. Even though all fractions possess vitamin-E activity, α-tocopherol possesses the highest vitamin-E activity, and is preferentially absorbed by receptor transfer protein in human liver (Traber and Sies 1996; Egesel et al. 2003). Among several genes involved in biosynthesis pathway, *vte4* (*γ-tocopherol methyl transferase*) gene on chromosome 5 was identified as the key gene that enhances the accumulation of α-tocopherol by converting γ-tocopherol (Li et al. 2012). Effect of *vte4* gene with higher accumulation of α-tocopherol was also reported by Lipka et al. (2013). Two insertion/deletions markers, *InDel7* and *InDel118* located in 5′UTR and promoter regions within *vte4* gene were identified to be significantly associated with higher level of α-tocopherol in maize kernels (Li et al. 2012; Das et al. 2018). Based on the *InDels*, four haplotypes viz., 0/0, 7/0, 0/118 and 7/118 in *vte4* were reported. Favourable haplotype (0/0: deletion at *InDel7* and *InDel118*) increases α-tocopherol by 2–3 folds over unfavourable haplotypes (7/118: insertion at *InDel7* and *InDel118*) (Li et al. 2012). Das et al. (2020) reported that the mean α-tocopherol of 0/0, 7/0 and 0/118 haplotypes among 54 diverse maize inbreds was much higher than the unfavourable (7/118) haplotype. Further, 0/0, 0/118 and 7/0 haplotypes possessed higher proportion of α-tocopherol/total tocopherol than 7/118 haplotype (Das et al. 2020). Das et al. (2019a) developed 36 maize hybrids by crossing inbreds possessing favourable haplotype (0/0). Majority of the experimental hybrids possessed significantly higher α-tocopherol (mean: 21.37 μg/g) than the check hybrids (mean: 11.16 μg/g). Das et al. (2019b) further identified one SNP (G to A) and three *InDels* in *vte4* gene which can further differentiate low and high α-tocopherol accumulating maize lines with favourable haplotypes. These newly reported *SNPs* and *InDels* along with *InDel118* and *InDel7* can be efficiently employed for the selection of favourable genotypes with higher levels of α-tocopherol in maize.

2.4 Improvement of Fe and Zn

Fe and Zn play a very important role in cellular functions, immune responses, reproductive health and other cerebral functions (Neeraja et al. 2017; Bhatt et al. 2018). Suboptimal consumption affects vital biological functions leading to reduced growth, cognitive response, reproductive performance and work productivity, besides significant socio-economic losses (Hallberg 1982; Sandstorm 1997). Over

60% of the world's population are deficient in Fe, while it is 30% for Zn (White and Broadley 2009). According to World Health Organization (WHO), 70% of the children under five and 56% the pregnant women in central and West Africa suffers from anaemia. Around 17% of the global population suffers from Zn deficiency (www.harvestplus.org). The target levels were set at 52 and 33 μg/g for Fe and Zn, respectively, depending on an estimated average requirement (EAR) of 1460 μg/day for Fe and 2960 μg/day for Zn (Bouis and Welch 2010; Andersson et al. 2017).

Substantial genetic variation for Zn has been identified in the tropical (Banziger and Long 2000; Chakraborti et al. 2011; Agrawal et al. 2012; Guleria et al. 2013) and temperate (Ahmadi et al. 1993; Brkic et al. 2003; Chen et al. 2007) germplasm including inbreds, landraces, hybrids and open-pollinated varieties. Using natural variation, eight maize cultivars rich in Zn have been released worldwide (Prasanna et al. 2020). However, lack of sufficient natural genetic variation, high genotype × environment effects and minor effects of the loci has limited the development of Fe rich maize cultivars (Abhijith et al. 2020). Understanding the physiological, biochemical and molecular mechanisms involved in the redistribution of Fe and Zn in the grain is essential for developing nutrient-fortified cultivars (Lin et al. 2009; Sperotto et al. 2010; Kobayashi and Nishizawa 2012). Several genes/candidate genes have been identified for metal uptake, transportation, xylem loading, remobilization and grain portioning in Arabidopsis, wheat, rice, maize, barley, maize and soybeans (Li et al. 2013; Lin et al. 2009; Sperotto et al. 2010; Grotz et al. 1998; Vert et al. 2001; Waters 2002). Forty-eight putative candidate genes have been reported to be responsible for the absorption, translocation and redistribution of Fe and Zn in the maize kernel (Sharma and Chauhan 2008). A study at IARI, New Delhi identified maize inbreds with favourable alleles of five candidate genes for Fe and at least six significantly associated genes for Zn (unpublished).

Several QTL (Table 6.2) were mapped in maize for the redistribution of Fe and Zn in the leaf, grain and bioavailability aspect (Simic et al. 2012; Lungaho et al. 2011; Qin et al. 2012; Baxter et al. 2013). Qin et al. (2012) reported the co-localized QTL for Fe and Zn on chromosomes 2, 7 and 9. In earlier research, the chromosomal bins 2.07 (Jin et al. 2013), 3.04–3.06 (Jin et al. 2013; Qin et al. 2012), 5.04 (Qin et al. 2012) and 9.06–9.07 (Jin et al. 2013) found several important QTL for Fe and Zn. In a recent study, 923 tropical/subtropical lines were genotyped through genotype-by-sequencing (GBS) and phenotyped at three locations and identified 46 SNPs (Fe-26 and Zn-20) through genome-wide association study (GWAS). A set of 11 SNPs each for Fe and Zn was validated in the bi-parental mapping population. Some of the SNPs explained relatively higher phenotypic variation (Hindu et al. 2018).

The introgression of QTL through MAS was found ineffective due to minor effects, genetic background variations and QTL × environment interactions (Bernardo 2016). Genomic selection (GS) has demonstrated its role in accelerating genetic gains in complex traits like Fe and Zn (Zhang et al. 2015; Crossa et al. 2017; Yuan et al. 2019). Velu et al. (2016) trained the genomic prediction model by using HarvestPlus-bred mapping panel (HPAM) with genomic prediction ability for Fe (0.324–0.734) and Zn (0.331–0.694) across different environments. The accuracy of genomic prediction has been tested in various maize populations using GBS markers

Table 6.2 QTL identified for kernel Fe and Zn in maize

S. no.	Parents	Mapping population	Fe		Zn		Reference(s)
			No. of QTL	R^2	No. of QTL	R^2	
1.	DH8 × DH40 and DH86 × S137	DH	8	10.2–43.7	9	9.4–48.8	Zhou et al. (2010)
2.	B84 × Os6–2	F_4	3	21.1	1	4.2	Simic et al. (2012)
3.	B73 × Mo17	IBM-RI	3	26.1	–	–	Lungaho et al. (2011)
4.	Mu6 × SDM and Mo17 × SDM	$F_{2:3}$	4	10.0–21.1	7	6.3–21.3	Qin et al. (2012)
5.	B73 × Mo17	IBM-RI	2	9.0–11.0	3	5–10	Baxter et al. (2013)
6.	178 × P53	$F_{2:3}$	1	16.9	4	5.9–17.6	Jin et al. (2013)
7.	178 × P53	RIL	8	3.2–5.4	20	2.8–16.8	Zhang et al. (2017)

and repeat amplification sequencing. Moderate to high genomic prediction ability (0.35–0.65) was observed for Zn content in maize by utilizing different populations and genotyping platforms (Prasanna et al. 2020). SNPs/haplotypes were identified and confirmed in the linkage and association mapping study in earlier generations, where population size was typically large, using the forward breeding method. To enhance genetic models' accuracy, GS can be introduced as a fixed effect in the GS models in the advanced generations or using the SNPs/haplotypes.

2.5 Reduction of Low Phytic Acid

Maize grains possess a higher concentration of phytic acid (PA), which is an anti-nutritional factor that drastically reduces the bioavailability of Fe and Zn, as the negative charge of phytic acid chelates the positively charged minerals (Castro-alba et al. 2019). Phytic acid is myo-inositol hexakisphosphate, and the primary storage compound of phosphorus in seeds that contributes about 80% of the total phosphorus in seed and as much as 1.5% of seed dry weight (Raboy et al. 2000). Thus, the reduction of PA in maize genotypes through genetic manipulation assumes great importance in enhancing the bioavailability of mineral elements. Besides, PA is also poorly digested in the monogastric animals limiting their growth, and their excreta possesses undigested phytic acid that increases the phosphorous in the environment leading to water pollution and a phenomenon called 'eutrophication' (Cromwell and Coffey 1991).

Bioavailability of Fe and Zn of maize grains is only 5% and 25% in human gut, respectively (Andersson et al. 2017). Thus, reduction of PA through genetic methods

is a feasible alternative (Abhijith et al. 2020). Low phytic acid (*lpa*) mutants, though reported in many crops, were isolated in maize for first time by Raboy et al. (2000). The phosphorous uptake and its subsequent transport to maturing seeds is not affected in these mutations, thus levels of total phosphorous remain nearly the same except for the decreased PA (Pilu et al. 2003). Several *lpa* mutants have been isolated in maize viz., *lpa1* (chromosome 1), *lpa2* (chromosome 1S), *lpa3* (chromosome 1S) and *lpa241* (chromosome 1). Pilu et al. (2003) reported a novel *lpa241* mutation which causes 90% reduction PA and possesses drastic effect on germination as compared to the wild types, hence may not be a viable choice. *lpa1-1* mutation leads to 55–65% reduction in PA in maize seeds and is due to mutation in trans-membrane transporter protein (MRP). The *lpa2-1* caused due to mutation in inositol phosphate kinase (IPK) enzyme leads to 50% reduction in PA (Raboy et al. 2000). No negative effect of *lpa1-1* and *lpa2-1* on seed germination and seedling vigour has been reported (Prasanna et al. 2020).

SNP-based marker for *lpa1-1* has been designed by Naidoo et al. (2012), while Sureshkumar et al. (2014) reported *umc2230* (SSR) for *lpa2-2*. Most recently, two dominant markers each specific to wild type and mutant allele based on the SNP (C to T transition) in *lpa1-1* gene sequence were reported (Abhijith et al. 2020). Abhijith et al. (2020) also developed a co-dominant cleaved amplified polymorphic sequence (CAPS) marker based on the transition mutation (A to G) discovered by comparing the full-length sequence of *lpa2-1* between the wild type and mutant allele. Both of these markers were also validated in several segregating generations. The *lpa2-2* was successfully introgressed into regionally well-adapted and productive elite inbred lines viz. UMI 395 and UMI 285 through MAS (Sureshkumar et al. 2014; Tamilkumar et al. 2014). Low PA-based maize inbreds and hybrids in the genetic background of *o2* and *crtRB1* genes have been developed (Bhatt et al. 2018). Several *lpa*-based inbreds have been developed from the F_2 segregants between normal and *lpa*-donor inbreds (Abhijith et al. 2020); and these lines were characterized for its agronomic performance, grain yield and nutritional quality (Ragi 2020). These newly developed low PA inbreds would serve as a potential germplasm for the development of low phytate maize hybrids.

3 Nutritional Improvement of Sweet Corn

Sweet corn (*Zea mays* var. *saccharata*) is a special type of corn with higher sugars in its endosperm compared to starch-rich endosperm of field corn (Hossain et al. 2015, 2019d; Chhabra et al. 2019a, b; Mehta et al. 2018). It is harvested at the kernel milky stage, generally 20–22 days after pollination (DAP) when kernels are of full size and exude milky liquid upon puncturing (Mehta et al. 2017a, b). Sweet corn is consumed in both fresh and processed form, and has become one of the preferred vegetables worldwide. Fresh sweet corn serves as an important ingredient in an array of soups and snacks items, and therefore become an integral part of diet in many South-East Asian countries (Feng et al. 2015). Moreover, roasted sweet corn ears are eaten as highly prized fresh product. Sweet corn being rich in fibre, minerals, antioxidants

and certain vitamins B possesses high nutritional value than field corn (Lertrat and Pulam 2007). The demand of sweet corn has increased in the last three decades and occupied a significant share in both global and domestic trade. Global import of frozen sweet corn is 385,296 tonnes with import value of US $448.93 million, while the same for preserved sweet corn is 786,859 tonnes with US $1020.77 million import value (FAOSTAT 2020). Global export of frozen and preserved sweet corn during the same year was 420,486 and 821,496 tonnes which valued over US $441.57 and US $1006.39, respectively.

In maize kernel, starch usually accounts for 73% of the kernel weight, of which ~25% is amylose and rest 75% is amylopectin (Whitt et al. 2002). Several recessive alleles such as *shrunken1* (*sh1*), *shrunken2* (*sh2*), *shrunken4* (*sh4*), *sugary1* (*su1*), *sugary2* (*su2*), *brittle1* (*bt1*), *brittle2* (*bt2*), *sugary enhancer1* (*se1*), *amylose extender1* (*ae1*), *waxy1* (*wx1*) and *dull1* (*du1*) have been identified which alters the content and composition of starch in maize endosperm (Boyer and Hannah 2001). Two recessive alleles viz., *su1* and *sh2* which limit the conversion of sugars into starch have been abundantly utilized in sweet corn breeding programmes (Lertrat and Pulam 2007). The sugar content of *sh2*-based sweet corn is about six-folds higher compared to ordinary maize, and popularly known as 'super sweet corn' or 'extra sweet corn' (Feng et al. 2008). Further, it retains the higher sugar and moisture content for longer time, thus varieties have extended shelf life. Sugary varieties (*su1su1*) accumulate three times more sugar and ten times more water-soluble phytoglycogen than field corn (Fisher and Boyer 1983). However, sugar level declines much faster compared to *sh2*-based sweet corn. *Sh2* codes the large subunit of ADP-pyrophosphorylase (AGPase), and is located on chromosome 3 and has been widely used in the breeding programmes globally (Mehta et al. 2017a, b; Chhabra et al. 2020). *Su1* gene is present on chromosome 4 and codes *Su1* isoamylase. Gene-based markers for *su1* (Chhabra et al. 2019b) and *sh2* (Chhabra et al. 2020) have been developed for their effective introgression through MAS.

However, traditional sweet corn being deficient in vitamin-A, vitamin-E, essential amino acids and anthocyanins does not contribute significantly in daily nutrient requirement (Yang et al. 2018). Biofortification of maize using both conventional and molecular breeding has resulted in the release of several biofortified varieties in field corn. However, very few studies have been conducted to enhance the nutritional quality of sweet corn (Table 6.1). Feng et al. (2015) developed biofortified sweet corn by marker-assisted backcrossing of *vte4* allele. The converted sweet corn lines possessed 19.72% higher α-tocopherols over the original sweet corn lines. O'Hare et al. (2015) through recombination breeding increased ZEA content in sweet corn from 0.2–0.3 mg/100 g of fresh weight (FW) to more than 2 mg/100 g of FW. Yang et al. (2018) increased the proA concentration from 1.55 to 3.95 μg/g in *lcyE* introgressed sweet corn lines. However, this increase in proA was very less than the target level of proA (14 μg/g) in maize. Mehta et al. (2020a) introgressed *o2* and *crtRB1* into parental lines of two sweet corn hybrids (ASKH-1 and ASKH-2), and reported higher concentration of proA (18.98 μg/g), lysine (0.39%) and tryptophan (0.10%) in improved hybrids compared to 3.12 μg/g, 0.23% and 0.06%, respectively in original hybrids. Jompuk et al. (2020) combined four genes viz., *sh2*, *Purple1*

(*Pr1*), *Coloured1* (*C1*) and *o2* into single genetic background, and observed that the improved genotypes had high sugar, 10-folds higher anthocyanin and 30% higher tryptophan. Mehta et al. (2020b) also reported that accumulation of lysine (0.367%, 0.345% and 0.315%), tryptophan (0.086%, 0.078% and 0.068%) and proA (21.32, 19.74 and 17.07 μg/g) in biofortified sweet corn was the highest at 20-DAP compared to 24- and 28-DAP, respectively.

4 Nutritional Improvement of Waxy Corn

Waxy maize or sticky maize (*Zea mays* var. *ceratina*) is used as a popular source of food especially in South-East Asian countries (Xiaoyang et al. 2017). Traditional maize accounts for approximately 75% amylopectin and 25% amylose, whereas waxy maize contains 95–100% of amylopectin by virtue of mutant *waxy1* (*wx1*) gene present on chromosome 9 (Zhou et al. 2016). Due to its varying compositions, food produced from waxy maize is easily digestible in human gut as compared to normal maize (Fukunaga et al. 2002). In addition, soft grains of waxy maize gain its popularity as a meal during breakfast owing to their cooking qualities and aroma (Fergason 2001). The viscous property is due to amylopectin, which makes it suitable for adhesive, paper and textile industries (Devi et al. 2017). *Wx1* gene codes the granule-bound starch synthase-1 (GBSS-I) enzyme, which catalyses amylose biosynthesis from ADP-glucose in amyloplasts of maize endosperm (Klosgen et al. 1986; Mason-Gamer et al. 1998). Different types of mutations such as insertion of transposon, retroposon and fragments of few nucleotides and deletion of nucleotides result in the mutant version of *wx1* and suppress the action of GBSS-I (Bao et al. 2012; Zhang et al. 2013).

Generally, the landraces of waxy maize are used as the main staple food for the ethnic groups located at far off areas (Swinkels and Turk 2006). Due to poverty and low educational level, these minority groups are unable to fulfil their balanced diet. Thus, biofortification of waxy maize with higher nutritional quality assumes great significance. Three SSRs viz., *phi022*, *phi027* and *phi061* and *InDel* marker *wx-2507F/RG* were used to identify favourable allele of *wx1* gene (Devi et al. 2017; Hossain et al. 2019c, d). Zhang et al. (2013) developed 18 waxy inbreds with as high as 27.06% more lysine than original parent lines. Similarly, Yang et al. (2013) pyramided *o16* and *wx1* genes in single genetic background and found 13 maize families, where lysine content was 16% more than the original parents. Similar trend of lysine improvement in waxy maize was also reported by Zhou et al. (2016). Talukder et al. (2019) have combined *wx1* and *o2* genes into four elite inbred lines HKI161, HKI163, HKI193-1 and HKI193-2. To improve the nutritional quality for waxy corn, Wang et al. (2019) integrated the *o2* genes into waxy line, QCL5013. Recently, Mishra et al. (2019) stacked *crtRB1, o2* and *wx1* alleles into parental inbreds of popular hybrids for simultaneous enhancement of lysine, tryptophan and proA (Table 6.1). Qi et al. (2020) created elite waxy inbred lines (94.9% high amylopectin) and hybrids using CRISPR-Cas9 editing technology.

5 Nutritional Improvement of Popcorn

Popcorn (*Zea mays* var. *everta*) is a whole grain familiar snack type of corn attributed to form large expanded flakes upon popping at sufficiently high temperature (Hoseney et al. 1983; Zunjare et al. 2015). Popcorn is relatively rich in fibre with nearly low glycemic index (low GI) index of 55, naturally low in fat and cholesterol free product with high economic value at consumers end. The global market for popcorn amounts to massive US $2.5 billion and expected to grow to US $6.22 billion by 2026 (https://www.fiormarkets.com). However, traditional popcorn is deficient in essential nutrients. Only few successful biofortification programmes improving the nutritional status of popcorn further are listed in Table 6.1. Adunola (2017) introgressed *o2* into the genetic background of popcorn, and reported higher tryptophan in the newly developed biofortified popcorn. Ren et al. (2018) also introgressed *o2* and selected for endosperm modification using vitreousness and high 27-kDa γ-zein content, and recovered high-lysine, fully poppable Quality Protein Popcorn (QPP). Lago et al. (2013) developed coloured popcorn variety rich in anthocyanins by selecting for *Pr1* and *Booster1* (*B1*). Microwave popping showed significant antioxidant capacity compared to colourless popcorn.

6 Impact of Biofortified Maize on Human Health

The nutritional significance of biofortified maize for infants and small children as well as adults has been demonstrated through systematic studies conducted worldwide (Teklewold et al. 2015). It has been observed that the biological value of QPM was equivalent to 90% of the milk protein (casein) as compared to just 40% of traditional maize (Prasanna et al. 2001). Children who consumed porridge prepared from QPM were relatively healthier than those who consumed porridge prepared using non-QPM. 12% growth rate of weight and 9% growth rate in height in infants and young children were observed when QPM diet was provided over diet made from traditional maize (Gunaratna et al. 2010). Further, children fed with QPM diet showed recovery from *Kwashiorkor.* The palatability and cooking quality of traditional food prepared from QPM were more acceptable due to its softness, perceived sweetness and longer shelf life in Eastern African countries (Akalu et al. 2010).

The health benefit of proA rich maize has also been well established through series of experiments (Bouis 2018). The provitamin A present in biofortified maize is more efficiently converted into retinol with conversion ratio of 2.8:1 (Howe and Tanumihardjo 2006), 3.2:1 (Muzhingi et al. 2011) and 6.5:1 (Li et al. 2010). In Zambia, the consumption of proA biofortified maize (PABM) diet enhanced serum xanthophylls and ^{13}C-natural abundance of retinol in children (Sheftel et al. 2017). Similarly, Palmer et al. (2018) in a study of 679 children in Zambia reported that consumption of PABM diet significantly improved serum β-carotene concentrations (0.273 μmol/L) compared to normal maize (0.147 μmol/L). Dube et al. (2018) reported that consumption of 200 g of proA-rich maize would provide at least 50% of RDA, thereby suggesting more efficient conversion ratio.

7 Impact of Biofortified Maize on Animal Growth and Development

Maize grain assumes importance as animal feed which contributes about 55–65% of the diet for poultry sector, and provides around 60% of energy, 30% of protein and 90% of starch in animals' diet (Dado 1999). Hence, the utility of quality protein maize (QPM) rich in lysine and tryptophan can be advantageous in the poultry and swine diet. The digestibility of cysteine (Gao 2002), lysine (Onimisi et al. 2008) and threonine (Panda et al. 2013) in poultry was higher when fed with QPM than non-QPM diet.

The feeding of QPM in broiler diets revealed higher weight gain (Osei et al. 1998; Nyanamba et al. 2003; Panda et al. 2013) as against non-QPM. The same results of dietary replacement on improved body weight gain and feed conversion ratio (FCR) in broilers were also shown by Bai (2002) and Amonelo and Roxas (2008). Onimisi et al. (2008) had replaced non-QPM diet at 0, 25, 50, 75 and 100% with QPM diets to Ross broiler chicks and compared their performance against a control (fed non-QPM diet + synthetic lysine). The results showed 100% dietary QPM increased body weight gain and FCR. The experiment of Mushipe et al. (2017) in Zimbabwe with similar method and objective revealed higher growth performance, feed efficiency and carcass yield of broiler chicken fed with QPM-based diet. The cost-saving for broiler feed has been reported by De Groote et al. (2010), Krishna et al. (2014) and Thapa et al. (2020).

It was also revealed that feeding QPM-based diet led to increased egg production (Osei et al. 1999; Zhai 2002). In White Leghorn layers, Panda et al. (2013) studied the utilization of QPM in the diet and found increased egg production and improved feed efficiency, while feed intake, egg weight, body weight gain and mortality remained unaffected. It was concluded that increased egg production with supplementation of lysine in non-QPM-based diet was comparable to QPM-based diet without supplemental lysine, thereby saving the cost of synthetic lysine (Tyagi et al. 2008; Panda et al. 2010). Moreover, feeding QPM-based diet improved yolk colour index (Zhai 2002; Panda et al. 2013). PABM has now emerged as an alternative to colour additives in the poultry industry (Diaz-Gomez et al. 2017). Chickens fed with PABM produced proA rich egg (Liu et al. 2012; Heying et al. 2014; Moreno et al. 2016; Sowa et al. 2017). The PABM fed chickens possessed higher redness and yellowness and lower lightness in the meat and skin colour. The PABM diet improved the skin and muscle colour of the 'Ovambo' chicken (Odunitan-Wayas et al. 2016).

The effect of QPM on pigs has also been well demonstrated. Burgoon et al. (1992) found that pigs fed with QPM possessed doubled the rate of weight-gain. In pigs, it has been observed that the dry matter intake, daily nitrogen intake, and digestible nitrogen intake was higher when fed with QPM, compared to those fed with non-QPM. Lysine digestibility was also higher in the QPM fed pigs than in the pigs fed with non-QPM diet (Mariscal-Landin et al. 2014). In piglets, Heying et al. (2014) found that consumption of proA carotenoids daily at the time of gestation and lactation increased the retinol status of liver.

8 Challenges and Future Prospects

Biofortified crops possess great potential to provide food and nutritional security. However, the area under biofortified crops is meagre compared to traditional varieties. There are several challenges for adoption and popularization of biofortified maize. There is a need to widen the genetic base of biofortified maize germplasm for the development of more diverse hybrids adaptable to various agro-ecologies. The biofortified germplasm should also possess adequate tolerance to major biotic and abiotic stresses to sustain high grain yield. Extensive demonstration of improved biofortified maize technologies in the farmers' field needs to be undertaken by conducting large scale on-farm demonstration. Quality seed production is the major issue for meeting the seed demand of biofortified maize. Participatory seed production programme including various stake holders needs to be developed for assuring quality seed availability. Awareness generation on the importance of biofortified food crops is also an important aspect of commercialization. It has now been well established that the biofortified varieties are *at par* with non-biofortified varieties for their yield potential, therefore yield inferiority should not be cause for non-adoption of biofortified varieties. The benefit of biofortified maize on human health has been well documented. Extension agencies should reach to the villagers for higher adoption of biofortified maize hybrids. Nutritious food is an important factor to the infants and young children for alleviating malnutrition. Family heads and especially mothers are the key to the adoption of biofortified maize as food in the family. Animal sector is also required to be sensitized on advantages of biofortified maize on poultry birds and pigs, and its subsequent net returns. Linkages with the poultry sector should be strengthened. Policy intervention is also necessary for the popularization of biofortified crops. The adoption of biofortified maize may be enhanced by supporting poultry and maize-based processed food industries through subsidies and loans. There is also a need of attaching the premium price to biofortified grains over available traditional maize. The protocol for the segregation of biofortified maize grains from normal corn in the markets needs to be standardized. Inclusion of biofortified foods in different government-sponsored schemes related to child and maternal nutrition would further help in alleviating the malnutrition.

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Pearl Millet: Biofortification Approaches in a Micronutrient Dense, Climate-Resilient Nutri-Cereal

7

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Abstract

Pearl millet forms an integral part of food and nutritional security to the millions resource-poor inhabitants in the semi-arid and arid regions of the world. Earlier considered as orphan, under used and neglected crop, pearl millet is the power-house of nutrients and has high resilience to harsh environments such as drought, salinity, and extreme temperature. It can yield well with limited resources. Micronutrient malnutrition "hidden hunger" continues to linger throughout the developing world particularly in the marginal environments where people cannot afford to have nutrient supplements in their diets. Hence improving the nutrient profile of native climate-resilient staple food crop like pearl millet can address the problem of micronutrient malnutrition. In this route, research was initiated on biofortification of grain micronutrients in pearl millet by ICRISAT and various NARS partners, resulted astounding progress in understanding the diversity and genetics of the traits, mapping and thereby devising a way to manipulate them for the development of high micronutrient-rich cultivars. Many of these cultivars became popular among farmers of India and Africa resulting in dynamic livelihood changes. The current chapter describes the success story of pearl millet biofortification program in context to micronutrient enrichment in grains.

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Pearl millet · Iron · Zinc · Biofortification · Bioavailability · Molecular mapping · QTLs

1 Introduction

The "Green Revolution" in India had helped to alleviate the crisis of food insufficiency by introducing high-yielding variety and modern tools and techniques. It has boosted agricultural production from 50 to 296.67 million metric tons (Nelson et al. 2019). India has reached a stage of surplus food production and is among the 15 leading exporters of agricultural commodities in the world with an export worth of US $38.54 billion in 2019 (Swaminathan 2013; APEDA 2020). As nutritional content in food being ignored and food accessibility did not improve over the years, nutritional intake remained disappointing leading to nutritious hunger. Even with such grain and food productivity, ~14% of the Indian population is undernourished. It also accounted for 35% of the world's stunted (low height-for-age) children under five and 17.3% of the world's wasted children (low weight for height). Almost 42% of adolescent girls aged 15–19 have a very low body mass index, while 54% found to be anemic (GHI 2020). Meanwhile, the financial crisis caused due to unemployment and pandemic has lead to increased volatility in food prices and further reduced the purchasing power of poor rural people. It was estimated that 50% increase in food price can lead to the prevalence of anemia by 25% in children and women (FAO et al. 2020). In this context, the relevance of biofortified crop products becomes essential. Biofortification is the concept of delivering micronutrients via staple foods through agronomic practices, conventional plant breeding, or modern genomics-assisted breeding. It has been recognized as the fifth most cost-effective investment by the Copenhagen Consensus (2008) in complimenting other existing interventions, such as supplementation and fortification, in fighting malnutrition (Meenakshi 2006). Biofortification provides an alternative to reach subgroups of the population where supplementation and conventional fortification activities are difficult to implement. These population groups often have limited purchasing power to access a nutrient-rich diverse diet (Hefferon 2016; Singh et al. 2016).

Pearl millet is one of the ideal staple food crops for biofortification. It is being cultivated over more than 26 million ha by 90 million farmers in arid and semi-arid regions of sub-Saharan Africa and the Indian subcontinent. Pearl millet makes the staple food of the majority of the resource-poor people and a source of cattle feed and fodder for livestock in the rainfed regions of the country. The crop naturally thrives well in marginal environments such as poor soil fertility, high pH, and Al^{3+} saturation and minimum moisture content, extreme temperature ranges, and salt stress (Varshney et al. 2017). Pearl millet grain produce is an important feedstock for several food products such as unleavened bread (roti or chapatti), porridge, gruel, and dessert which is referred to as a "poor man's bread" (Burton et al. 1972). Pearl

millet flour is one of the good substitutes of wheat flour in whole-grain bread, pretzels, crackers, and tortillas (Dahlberg et al. 2003). In the future under climate change and increasing temperature with probable low availability of irrigation water and erratic rainfall, millets will become important crops for food and nutrient security in the world.

Micronutrient malnutrition has been recognized as a big challenge to human health emerged due to dietary deficiency of mineral nutrients in sub-Saharan Africa and the Indian sub-continent. Among all the micronutrients, iron and zinc deficiencies are the most striking and more than 2 billion individuals or one in three people are suffering from Fe deficiency alone and the number of Zn deficiency are also close (FAO 2003). Micronutrient malnutrition is widely termed as the 'hidden hunger' primarily attributed to Fe and Zn deficiencies leading to devastating health problems (Stein 2010). Pearl millet is a highly nutritious cereal with higher Fe than other cereals and endowed with vast diversity for micronutrients, which has harvested in improvement for grain iron (GFe) and zinc (GZn) contents with high yield and other agronomic traits (Mahendrakar et al. 2019; Govindaraj et al. 2019). Dietary diversification, food supplementation, fortification, and biofortification of crop plants have been recommended to address micronutrient malnutrition, to that micronutrients can be availed by deprived people survive solely on plant-based staple foods (Stein 2010; Saltzman et al. 2013). Among all these approaches, biofortification is the most successful strategy to breed such nutrient-rich varieties of staple food crops (Anuradha et al. 2017a, b). Biofortified pearl millet can contribute to enhanced Fe and Zn intake, especially in marginal and low-income areas where availability of the nutrient-rich diversified foods is limited and/or unaffordable. Biofortified pearl millet lines can be developed using marker-assisted breeding approaches. Biofortified food is inexpensive, sustainable, and affordable to poor people (Bouis and Welch 2010). It indicates the potentials of biofortified pearl millet to combat malnutrition and malnutrition and hidden hunger.

2 Importance of Pearl Millet Vis-a-Vis Other Cereals in the Human Diet

Pearl millet is naturally blessed with several nutritional properties in comparison to other staple cereals. It is an excellent source of organic (i.e. carbohydrates, proteins, fat, dietary fibers, vitamins) as well as inorganic (Fe, Zn, etc.) nutrients and a cost-effective source of energy required by the human to meet their dietary requirement. Pearl millet is a rich source of energy (360 kcal/100 g) comparable with sorghum, wheat, rice, and maize (Table 7.1). Carbohydrate content of pearl millet is 67.5 g/100 g; with 60–70% starch comprising 28.8–31.9% amylose among which 14.6–17.2% form complex with native lipids. It encompasses a comparatively higher water absorption capacity and swelling index than the other cereal starches (Lestienne et al. 2007). Free sugars such as sucrose, glucose, fructose, and raffinose make 2.6–2.8% of total carbohydrates in pearl millet grains. Pearl millet contains an adequate amount of dietary fibers (1.2 g/100 g) among which most of them belong to

Table 7.1 Nutrient composition of major cereals (per 100 g) edible portion

Contents	Pearl millet	Sorghum	Maize	Rice	Wheat
A. Proximate composition					
Moisture (%)	12.40	11.90	14.90	13.00	12.80
Protein (%)	11.60	10.40	11.10	6.90	11.80
Fat (%)	5.00	1.90	3.60	0.40	0.90
Fiber (%)	1.20	1.60	2.70	0.20	0.30
Carbohydrates (%)	67.50	72.40	66.20	79.20	74.10
Calories (kcal)	360.00	349.00	342.00	348.00	349.00
B. Micronutrients					
Calcium (%)	0.05	0.03	0.01	0.01	0.02
Phosphorous (%)	0.35	0.28	0.33	0.28	0.09
Iron (mg)	8.80	6.20	2.20	2.80	1.00
Magnesium (mg)	125.00	140.00	144.00	48.00	139.00
Copper (mg)	0.55	0.55	0.19	0.72	0.49
Sodium (mg)	10.00	7.00	6.00	3.00	18.00
Potassium (mg)	402.00	321.00	290.00	110.00	349.00
Zinc (mg)	3.1	1.7	2.2	1.3	2.7
Carotene (mg)	132.00	47.00	90.00	9.00	29.00
Thiamine (mg)	0.33	0.37	0.42	0.27	0.12
Riboflavin (mg)	0.25	0.13	0.10	0.12	0.07
Niacin (mg)	2.30	1.80	1.40	4.00	1.20
Folic acid (μg)	45.5	20	–	8	36.6
C. Amino acids + (g/16 g N)					
Tryptophan	1.74	1.10	0.60	1.00	1.20
Threonine	3.92	3.60	4.00	3.70	2.70
Lysine	3.01	2.70	2.90	3.80	2.60
Methionine	1.82	1.70	1.90	1.70	1.40
Isoleucine	4.78	5.40	4.60	4.50	4.10
Leucine	10.71	16.10	13.40	8.20	6.30
Cysteine	1.03	1.70	1.30	1.30	2.10
Phenylalanine	5.37	5.00	4.50	4.80	4.60
Valine	5.73	5.70	5.10	6.70	4.30
Arginine	5.14	3.80	3.50	5.50	4.50
Histidine	2.32	1.90	2.60	1.60	1.90
Tyrosine	3.41	2.80	6.10	4.40	3.50

Source: Gopalan et al. (1999, 2004), Taylor (2004), Bashir et al. (2014) and Minnis-Ndimba et al. (2015)

an insoluble form when compared with other grains (National Institute of Nutrition 2017). Pearl millet is having a low glycemic index score of 55 and significantly rich in resistant starch. Hence pearl millet can be an excellent diet for diabetics, obese, and celiac disease patients.

The amino acid profile of pearl millet protein includes most of the essential amino acids, which is comparatively higher than wheat and maize proteins (Table 7.1).

With low prolamin fraction, pearl millet is a gluten-free grain and is the only grain that retains its alkaline properties after being cooked which is ideal for people with gluten allergy. Pearl millet is rich in unsaturated fatty acids with a higher content of nutritionally important omega-3 fatty acids than other cereal grains. Pearl millet is an adequate source of dietary minerals such as Fe, Zn, phosphorus, calcium, magnesium, and copper (Serna-Saldivar 2016). However, like all cereals, pearl millet contains phytate, an anti-nutrient that chelates with minerals forming complexes hence reducing their effective absorption and utilization by humans (Kent 1994). Pearl millet flour is rich in brain cell promoting factors that can alleviate Parkinson's disease such as gallic acid, chlorogenic acid, syringic acid, *p*-coumaric acid, and others.

2.1 Importance of Fe and Zn in Human Health

Fe and Zn deficiencies rank 9th and 11th, respectively, among the top 20 risk factors contributing to the global burden of disease (Stein 2010). Deficiency of Fe in the diet leads to anemia (Gregory et al. 2017), and also causes stunted growth, low birth weight, and delayed mental development (Singhal et al. 2018). Likewise, Zn deficiency causes hypogonadism, dwarfism and geophagia, and childhood mortality. Prolonged Zn deficiency leads to increased susceptibility to infectious diseases such as pneumonia, diarrhoea, reduced physical performance and work productivity, and poor birth outcomes in pregnant women (Cakmak and Kutman 2018). Biofortified pearl millet with high GFe and GZn levels serves as the logical vehicle for providing minerals in the diets of the people to alleviate micronutrient deficiencies (Govindaraj et al. 2019).

3 Development of Elite Germplasm and Breeding Lines for High Grain Iron and Zinc Content

In genetic biofortification, plant breeding techniques are used to develop food crops with higher micronutrient levels, reducing levels of anti-nutrients and increasing the levels of substances that promote nutrient absorption in addition to higher yield (Bouis et al. 2011). In this context, the first step is to screen existing accessions in germplasm collection for sufficient genetic variation to breed for a particular trait. HarvestPlus program has set needed levels for GFe, GZn, and provitamin A carotenoids in target crops after addressing these issues. In case of pearl millet HarvestPlus target level is a minimum 78 ppm of GFe in parental lines. Secondly, the identification of highly heterotic combinations with minimum set targets of micronutrients or breeding for increased bioavailability. In pearl millet, the bioavailability of Fe can be assumed to be 7.0–7.5% (Govindaraj et al. 2019). A major initiative toward the development of high-iron pearl millet cultivars has been taken by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and National Agricultural Research System (NARS) partners. The development of screening techniques, the extent of genetic variation for grain micronutrient and

bioavailability contents in germplasm collection, genetics of traits, nature of genotype × environment interaction, and relationships between grain minerals and agronomic traits would determine breeding efficiency for developing high yield cultivars with enhanced nutrient content. Therefore, detailed insight is provided here to assess the progress made so far in these areas.

3.1 Screening of Pearl Millet Lines

The availability of low cost and quick throughput but sensitive analytical methods for micronutrient screening is a prerequisite for successful biofortification breeding. Several destructive methods such as atomic absorption spectrometry (AAS) and inductively coupled plasma optical emission spectrometry (ICP) are available for mineral analysis. To screen thousands of germplasm lines, simple, rapid, and time and cost-efficient approaches are used to enhance the breeding efficiency. X-ray fluorescence spectrometer (XRF) and near-infrared reflectance spectroscopy (NIRS) are non-destructive rapid method preferred to assess the organic compounds which are indirectly related to inorganic elements in grain samples (Osborne 2006). The efficiency of XRF over ICP for high-throughput Fe and Zn estimation in pearl millet grain was demonstrated with large samples from several trials (Govindaraj et al. 2016a, b).

3.2 Assessment of Genetic Diversity

A huge range of genetic variability exists in pearl millet germplasm for GFe and GZn content, which has been reflected in the outcomes of experiments conducted at ICRISAT and various NARS centers in India. Several studies have been conducted by different research groups around the world to assess the genetic diversity for GFe and GZn content from time to time using different sets of germplasm accessions and breeding lines (Table 7.2).

3.3 Genetics of Traits and Nature of Gene Action

Understanding the nature of gene action and inheritance patterns of grain micronutrient is crucial to develop effective biofortification breeding strategies. Several studies in pearl millet using different mating designs showed that the inheritance of GFe and GZn contents is largely attributed to additive genetic variance with a higher magnitude of heritability, explaining the simple inheritance pattern and simple selection for micronutrients (Velu 2006; Arulselvi et al. 2007; Gupta et al. 2009; Govindaraj et al. 2016a, b). In general, variability among the hybrids attributable to general combining ability (σ^2GCA) was 3–4 times greater than the variability attributable to specific combining ability (σ^2SCA) for GFe and GZn contents. This indicated that the GCA effect for both Fe and Zn contents were predominantly under additive genetic control in pearl millet (Velu et al. 2011; Rai et al. 2012; Govindaraj

Table 7.2 Range of grain concentrations for Fe and Zn in contemporary pearl millet lines

Sl. no.	Material type	No. of entries	Fe range (mg/kg)	Mean Fe (mg/kg)	Zn range (mg/kg)	Mean Zn (mg/kg)	Reference
1.	Released cultivars	27	40–581	169	10–66	40	Jambunathan and Subramanian (1988)
2.	Breeding lines	120	30.1–75.7	49.5	24.5–64.8	43.9	Velu et al. (2007)
3.	Advanced breeding lines	386	18–97		22–69		Rai et al. (2012)
	Population progenies	232	52–135		40–92		
4.	Population trials	16	51–121		46–87		Rai et al. (2014)
5.	Designated B-lines	14	30.3–77.2		27.4–45.3		Kanatti et al. (2014)
	Designated R-lines	14	32.0–82.1		29.0–55.5		
6.	Landrace and local cultivars of Sudan	225	19.7–86.4	42.9	13.5–82.4	40.3	Bashir et al. (2014)
7.	OPV	18	42–67		37–52		Rai et al. (2016)
	Hybrids	122	46–56		37–44		
8.	F_6-RILs mapping population (selfed seed)	144	28.4–124.0	68.48	28.7–119.8	64.21	Kumar et al. (2016)
	F_6-RILs mapping population (OP seeds)		22.4–77.4	45.68	21.9–73.7	45.72	
9.	Indian GWAS panel	130	32.30–111.90	58.95	26.62–73.68	42.62	Anuradha et al. (2017a, b)
10.	F_6-RILs mapping population	317	20.0–131.0	54	18.2–109.8	43.9	Kumar et al. (2018)
11.	Commercial cultivars in India	122	31–73	45	32–55	41	Govindaraj et al. (2020)

et al. 2013; Kanatti et al. 2014). Highly significant and positive correlations between hybrid performance per se and mid-parental values provided further support for these micronutrients being largely under additive genetic control. In the contrary, another study reported a predominance of non-additive genetic variance for these micronutrients (Arulselvi et al. 2006) and the presence of a duplicate type of epistasis along with additive gene effect for grain iron content (Gaoh et al. 2020). The differences between the direct crosses and reciprocal crosses were non-significant both for the Fe and Zn contents both in genotypes with high- and low-content genetic backgrounds (Kanatti et al. 2019). This indicated that genetics of both the micronutrients are controlled by nuclear determinants of male and female parents which showed the relatively greater importance of both nuclear than cytoplasmic contribution. Further, genetic studies revealed the high grain Fe and Zn parents had positive and significant GCA effects, while parents with low grain Fe and Zn had significant negative GCA effects (Govindaraj et al. 2013; Kanatti et al. 2014). This pattern of genetic control suggested that the selection for higher grain micronutrients should be commenced in an earlier generation while agronomic superiority can be selected in later generations.

3.4 Genotype Environment Interaction (GEI)

Genotype by environment interactions (G × E) are a major obstacle for developing micronutrient-rich pearl millet hybrids for a particular zone. Micronutrient status in the soil varies greatly in drylands where pearl millet cultivation is concentrated. Also, plant-associated micro-climate and microbiome also can stimulate growth and influence yield and quality of edible parts by affecting nutrient mobilization and transport (Pii et al. 2016). Under such conditions, G × E interaction for agronomic and grain nutrient traits is expected to be large and may not permit differentiation of performance of genotypes across environments (Satyavathi et al. 2015; Anuradha et al. 2017a, b). Various studies were reported to show a significant role of environment and G × E determining the levels of GFe and GZn contents in pearl millet. Such studies also identified donors that are high and stable micronutrient contents (Satyavathi et al. 2015; Rai et al. 2016; Pawar et al. 2018; Singhal et al. 2018, 2019). Some GXE studies showed that GZn content is much more sensitive to GEI when compared to GFe content (Singhal et al. 2018; Anuradha et al. 2017a, b). There were regular experiments such as high Fe progeny trials (HFePT) and consortia research project on biofortification-parental trials (CRPB-PLT) carried out by ICRISAT and NARS under AICPMIP all over India to screen high iron and zinc-rich advanced pearl millet breeding lines.

3.5 Association with Agronomic Traits

Understanding the various associations among traits is important in plant breeding because it quantifies the degree of genetic and non-genetic association between two or more traits, allowing the indirect selection (Hallauer and Miranda Filho 1988).

There are various reports which suggest a highly positive correlation between iron and zinc (>0.70, *p*-value <0.01) indicating the effectiveness of simultaneous selection for GFe and GZn in pearl millet (Rai et al. 2016; Anuradha et al. 2017a, b; Kumar et al. 2018; Singhal et al. 2018). Recently, Govindaraj et al. (2020) investigated macro- and micro-elemental profiles in popular Indian cultivars, indicated the existence of great potential for the concurrent improvement of GFe and GZn without lowering the other grain minerals, as other micronutrients except sulfur were not associated with both the traits. There were many promoters and inhibitors which can influence nutrient bioavailability in pearl millet (Krishnan and Meera 2017). Phytic acid has been reported to be the major inhibitory factor, insoluble fiber also forms fiber-phytate-mineral complexes (Lestienne et al. 2005). Polyphenols also form insoluble complexes with iron and cause inhibition of iron absorption (Brune et al. 1991; Cercamondi et al. 2014). Studies also pointed out that certain grain components like vitamin A and β-carotene acted as enhancers by binding to iron, keeping it in the soluble form in the intestinal lumen, thus preventing the phytic acid and polyphenols inhibiting iron absorption (García-Casal et al. 1998). The Fe and Zn contents had a negative and mostly non-significant correlation with grain yield in pearl millet (Rai et al. 2016; Kanatti et al. 2014; Yadav et al. 2016).

3.6 Development of Molecular Markers and Identification of Marker Trait Association

During the first decade of twenty-first century, use of molecular markers in pearl millet genetics and breeding has made some headway, and pearl millet has been promoted to the status of a molecular crop through a series of collaborative projects involving the John Innes Centre (JIC), ICRISAT and their collaborators supported by the Plant Sciences Research Program of the UK's Department for International Development (DFID) (Gale et al. 2005). Several DNA-based molecular markers have been developed and exploited in genetic diversity, QTLs/genes identification, and marker-aided breeding for faster pearl millet breeding (Kumar et al. 2018; Srivastava et al. 2020a, b). A flanking marker associated with target trait is an important tool for nutritional-enrichment breeding strategy. Marker-linked traits can considerably save time, resources, and effort to bring multiple favorable alleles or genes when they are governed by multiple genes (Manwaring et al. 2016). Pearl millet is one of the crops where marker-assisted breeding (MAB) strategies have been applied to develop downy mildew-resistant variety "Improved HHB 67" (Hash et al. 2003). There are many efforts to discover, validate, and deploy trait-based molecular markers for grain iron and zinc content in pearl millet (Kumar et al. 2016, 2018; Anuradha et al. 2017a, b) whose details are given in Table 7.3. Most of these studies identified genes or QTLs with individually showing very low phenotypic effects. Hence the favorable alleles identified need to validate through a meta-analysis in a single background. Also upon validation, these favorable alleles can put together in elite materials through a marker-assisted recurrent selection (MARS) (Bernardo and Charcosset 2006). Various programs are still undergoing in ICRISAT to introgress identified QTLs into elite populations and hybrids.

Table 7.3 Review of various QTLs and association mapping studies carried out in reference to grain Fe and Zn content in pearl millet

Author	Objective	Materials used	No. of lines evaluated	GFe range (ppm)	GZn range (ppm)	No. of markers used	No. of associated marker/s or QTL/s with LG	Flanking markers	PVE
Kumar et al. (2016)	Mapping quantitative trait loci (QTLs) controlling high iron and zinc content in self and open pollinated grains of pearl millet	F_6 recombinant inbred lines (RIL) derived between ICMB 841-P3 (Low Fe-Zn) × 863B-P2 (high Fe-Zn).	144	(a) Fe-Self 28.4–124 (b) Fe-OP 22.4–77.4	(a) Zn-Self 28.7–119.8 (b) Zn-OP 21.9–73.7	96 SSRs; 208 DArT	(a) In selfed seed, a single co-localized QTLs for Fe and Zn content on LG 3 (b) In open pollinated seeds—two QTLs for grain Fe content on LG 3 and 5, and two QTLs for grain Zn content on LG 3 and LG 7.	Fe and Zn: Xpsmp2214-Xipes0142. OP Fe: Xpsmp2214-Xipes0142 Pgbp5908-Pgpb6674 OP Zn: Xpsmp2214-Xipes0142. Xpsmp2040-Pgpb10727	Fe-Self: 19%, Zn-Self: 36%, Fe-OP: 16%, Zn-OP: 42%
Anuradha et al. (2017)	Genome-wide association mapping of QTLs for Fe and Zn	A diverse inbred panel consists of B lines, R lines and advanced breeding lines	130	32.3–111.9	26.6–73.7	250 SSRs and 17 genic markers	SSR markers were associated with both grain Fe and Zn content were present on LG 3, LG 5 and LG 7.	Xipes 0180 (aspartic proteinase gene) Xpsmp2261 (intergenic region) Xipes 0096 (dropped)	11.40%, 13.34% and 11.38% R^2 value respectively.

Kumar et al. (2018)	Mapping grain iron and zinc content QTLs in an iniadi-derived immortal population of pearl millet	F_6-RILs derived between (ICMS 8511-S1-17-2-1-1-B-P03: Low Fe-Zn × AIMP 92901-S1-183-2-2-B-08): High Fe-Zn	317	20–131	18–110	177 DArTs and 19 SSRs	Eleven QTLs were for Fe and eight were for Zn. Three co-mapped QTLs for Fe and Zn were observed, one on LG1 and two on LG7	Fe-Zn: pgpb10531-pgpb9130, Fe: pgpb8427-pgpb13221 pgpb11938-pgpb8987 Zn: Xipes198-pgpb8427 pgpb12329-pgpb9721	Fe: 31.9% Zn: 30.4% Fe: 12.2%, 12.5% Zn: 10.2%, 10.9% respectively

4 Cultivar Development Through Conventional and Molecular Approaches

4.1 Fast Track Breeding Approach for Biofortified Hybrid and OPV Development

Earlier in India, OPVs were most popular due to its genetic plasticity toward adverse conditions which has been replaced by high-yielding uniform heterotic single cross hybrids. Breeding for a biofortified hybrid is entirely different in the case of breeding for a biofortified OPV (Fig. 7.1). The extent of genetic variation is very critical for both hybrids and OPVs to initiate a breeding program aimed at trait-specific breeding. The assessment of micro-nutrient variation was undertaken using phenotyping protocols, as described earlier. As these traits were governed by additive gene action and their heritability were relatively high (Velu et al. 2011; Govindaraj et al. 2013; Kanatti et al. 2014; Govindaraj et al. 2016b; Anuradha et al. 2017a, b; Singhal et al. 2018, 2019), the pedigree method of breeding was deployed for progenies derived from primarily biparental crosses, each or both having high micronutrient profile and desired agronomic score (Satyavathi et al. 2015). There are various donors available which were environmentally stable and high performing such as iniadi germplasm (Satyavathi et al. 2015; Rai et al. 2016). Since, Fe/Zn have preponderance of additive

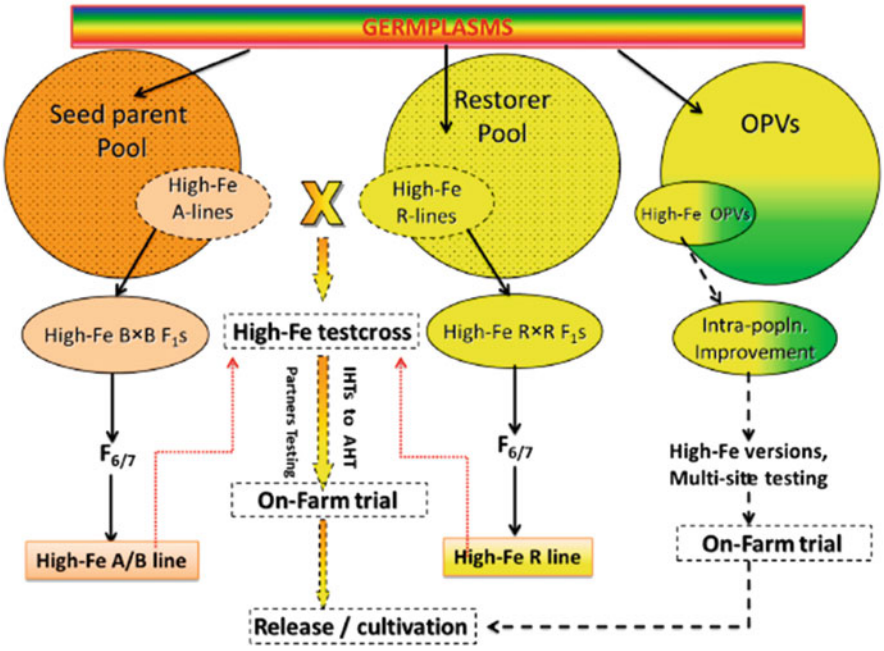

Fig. 7.1 Schematic representation of fast track breeding approach followed in ICRISAT for the development of high yielding Fe-Zn rich hybrids and OPVs. (Courtesy: Govindaraj et al. 2019)

gene effects, hybrid parental lines should be bred separately as two different pools for high micronutrient density, requiring a separate hybrid parent-development program (Govindaraj et al. 2019). Considering and maintaining a genetic distance between hybrid parental pools helps us to realize higher heterosis (Ramya et al. 2018; Singh and Gupta 2019). These progenies will be selected for high micronutrients along with a good agronomic score will be further evaluated in multilocation trials covering at least 10–12 pearl millet growing locations for GEI on Fe/Zn and lines showing consistent higher performance will be made available to both the public and private R&D sectors which are actively engaged in breeding pearl millet hybrids. In this direction, 174 high-Fe early-generation progenies (B × B progenies and R × R progenies) have been developed, which in trials conducted at Patancheru had shown >90 mg/kg Fe density and 36–72 mg/kg Zn density (Govindaraj et al. 2019). In case of OPVs, assessment of micro-nutrient variation within line was undertaken, in moderate/higher Fe/Zn content breeding lines or in a population that is not deliberately bred for the micronutrient content. Those progenies lines showing higher levels of micronutrients were pooled together. They were allowed to mate in isolation over generation with repeated selection of superior performing progenies, so that favorable genes for the target trait got accumulated. This will serve as an improved OPV developed with short/medium objective for rapid delivery high-Fe cultivars.

4.2 Current Status of Biofortified Cultivar and Its Adoption

With the efforts of ICRISAT and NARS partners, a handful of biofortified varieties and hybrids have been released by Govt. of India for cultivation. Rai et al. (2016) screened around 18 OPVs and 122 hybrids released and/or commercialized in India. Among OPVs, ICTP 8203 released in 1988 (Fe: 67 mg/kg and Zn: 52 mg/kg) and ICMV 221 in 1993 (Fe: 61 mg/kg and Zn: 45 mg/kg) were found promising for high Fe-Zn content. While, among hybrids Ajeet 38, Proagro XL 51, PAC 903, and 86M86 have been developed with an Fe content of 55–56 mg/kg and Zn content of 39–41 mg/kg. First systematic breeding effort to develop a high Fe cultivar resulted in a world first high-Fe pearl millet variety 'Dhanashakti' was developed by utilizing the intra-population variability within ICTP 8203, an early-maturing, large-seeded, disease resistant, and high-yielding open-pollinated variety (OPV), was released in 2014. The improved version of variety ICTP 8203,has Fe content of 71 mg/kg without any change in Zn content. Likewise, variety ICMV 221Fe11-2, a better version of variety ICMV 221, has been developed with high Fe (81 mg/kg) and Zn (51 mg/kg) content. Hybrids ICMH 1201 and ICMH 1301 have been developed at ICRISAT with Fe content of 75 and 77 mg/kg, respectively. Biofortified pearl millet hybrid HHB 299 was developed by CCSHAU, Hisar with an Fe content of 73 ppm and average grain yield of 39.5 q/ha, which was notified in 2018 (AICPMIP 2020). Also, biofortified hybrid AHB 1200 Fe has been notified and four other biofortified hybrids, RHB 233, RHB 234, HHB 311, and AHB 1269, have been released during 2018 (Table 7.4).

Table 7.4 List and salient features of biofortified cultivars developed and in cultivation in India

Sl. no.	Hybrid/ variety	Breeding station	Notification details	Area of adoption	Salient features	DF	DM	Grain yield (kg/ha)	Fodder yield (q/ha)	Grain Fe content (ppm)	Grain zinc content (ppm)
1.	Dhanshakti	ICRISAT, India and MPKV, Dhule	S. O 1146 (E) 24.04.2014	Maharashtra, Karnataka, AP, Tamil Nadu, Rajasthan, Haryana, MP, Gujarat, UP and Punjab.	Early maturing bold, globular, shining slate grey colored seed resistant to downy mildew disease.	45	76	2199	53	81	43
2.	HHB 299	CCS HAU, Hisar	S. O 1379 (E) 27.03.2018	Rajasthan, Haryana, Gujarat, Punjab, Delhi, Maharashtra and Tamil Nadu.	Medium maturing, compact panicle grayish hexagonal shaped grain and resistant to major diseases.	50	81	3274	73	73	41
3.	AHB 1200	NARP, Aurangabad	S. O 1379 (E) 27.03.2018	Rajasthan, Haryana, Gujarat, Punjab, Delhi, Maharashtra and Tamil Nadu, AP and Telangana	Medium maturing, cylindrical panicle resistant to downy mildew and highly responsive to fertilizers.	47	78	3170	70	77	39
4.	AHB 1269	NARP, Aurangabad	S. O 1498 (E) 01.04.2019	Rajasthan, Haryana, Gujarat, Punjab, UP, Delhi, Maharashtra,	Medium maturing, long cylindrical type panicle, bold	50	81	3168	74	91	43

				Tamil Nadu, AP, Telangana and Karnataka.	grain and resistant to major diseases.						
5.	HHB 311	CCS HAU, Hisar	S. O 3220 (E) 06.09.2019	Rajasthan, Haryana, Gujarat, MP, Punjab, Delhi, Maharashtra and Tamil Nadu	Medium maturing, compact panicle having grey colored hexagonal shaped grain, highly resistant to downy mildew and other diseases.	50	81	3173	72	83	39
6.	RHB 233	SKNAU, Jobner	S. O 3220 (E) 06.09.2019	Rajasthan, Haryana, Gujarat, MP, Punjab, Delhi, Maharashtra and Tamil Nadu	Medium maturing, grey globular shaped grain; highly resistant to blast and downy mildew diseases.	49	80	3157	74	83	46
7.	RHB 234	SKN AU, Jobner	S. O 3220 (E) 06.09.2019	Rajasthan, Haryana, Gujarat, MP, Punjab, Delhi, Maharashtra and Tamil Nadu	Medium maturing, conical shaped compact panicle with grayish colored hexagonal shaped grain, highly resistant to downy mildew.	49	81	3169	71	84	41

ICAR-AICRP on Pearl millet (AICPMIP) constructed a special module to test and release biofortified pearl millet cultivars in India (AICPMIP 2018). Furthermore, ICAR has endorsed a landmark decision on the inclusion of the minimum levels of iron (42 ppm) and zinc (32 ppm) in varietal promotion criteria for future pearl millet varieties to be released in the country which is the first of its kind in the world. Thus, along with yield improvement, focus on the nutritional improvement was also taken care of in pearl millet and in order to develop biofortified varieties/hybrids with enhanced Fe and Zn content. Visionary breeding tactics, combined with appropriate governmental intervention, can result in significantly better progress toward the adoption of high-Fe hybrids with high grain production gains.

5 Conclusion and Way Forward

Pearl millet can be rightly called as the crop of the future by virtue of its ability to grow profitably under harsh weather and soil conditions. Grain micronutrient, particularly Fe and Zn content, has been an area of active research in the fields of genomics, mapping, and cultivar development through conventional and molecular breeding approaches. The efforts have resulted in the identification of QTLs, alleles, and candidate genes on the genomics side and the development of OPVs and single-cross hybrids on the plant breeding side. Many of the cultivars released in India and Africa are becoming popular with the farmers. With the advent of new genetic and genomic tools in pearl millet, it will soon be possible to integrate Fe and Zn traits in whole-genome selection-based cultivar development schemes.

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Biofortifying Sorghum for Delivering Grain Micronutrients in High Yielding Cultivars with Market-Preferred Traits

8

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Abstract

Micronutrient malnutrition, particularly among women and children, is one of the greatest global challenges of our times and the national Governments and international organizations are following various approaches to combat it. Biofortification—increasing the micronutrient density in edible plant parts by genetic means, is one of the cost-effective and sustainable methods to address micronutrient malnutrition. Sorghum is one of the major staples globally and it meets more than 50% of micronutrient requirements of low-income group populations in predominantly sorghum eating areas. We developed biofortified sorghums with elevated levels of grain Fe and Zn combined with higher grain yield possessing farmer and market-preferred grain and stover traits. The first biofortified sorghum cultivar "Parbhani Shakti" was released in India in 2018, which, besides high Fe and Zn, has higher protein content and lower phytates content. An innovative "Seed Consortium" was built to take this variety to the farmers in the shortest possible time to benefit the farmers and consumers. Multi-stakeholder partnership was the key in this endeavor and Indian NARS, farmers,

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public sector seed organizations; media and government played a key role along with ICRISAT. We describe this journey and learnings in brief in this chapter.

Keywords

Biofortification · Grain iron · Grain zinc · Seed consortium · Sorghum

1 Introduction

Micronutrient (MN) malnutrition is one of the greatest global challenges of our times and developing countries in Africa and South Asia are highly affected with the highest concentration of micronutrient malnourished people. Micronutrients (MNs) are essential for living organisms, which are limiting in many diets, particularly in the low-income group populations, predominantly in South Asia and sub-Saharan Africa. MNs, although only required by the body in small amounts, are vital for development, disease prevention, and well-being. Micronutrients are not produced in the body and must be derived from the diet. Deficiencies in micronutrients such as iron, iodine, vitamin A, folate, and zinc can have devastating consequences. At least half of the children worldwide ages 6 months to 5 years suffer from one or more micronutrient deficiencies, and globally more than two billion people are affected.

Iron (Fe) is an essential mineral critical for motor and cognitive development. Children and pregnant women are especially vulnerable to the consequences of iron deficiency. Low hemoglobin concentration (anemia) affects 43% of children 5 years of age and 38% of pregnant women globally (Stevens et al. 2013). Flour fortification with iron and folic acid is globally recognized as one of the most effective and low-cost micronutrient interventions (Copenhagen Consensus 2012).

Zinc (Zn) is a mineral that promotes immunity, resistance to infection, and proper growth and development of the nervous system, and is integral to healthy pregnancy outcomes. Nearly 17.3% of the global population is at risk for zinc deficiency due to dietary inadequacy, though upto 30% of people are at risk in some regions of the world (Wessels et al. 2013). Zinc supplementation reduces the incidence of premature birth, decreases childhood diarrhea and respiratory infections, lowers all-cause mortality, and increases growth and weight gain among infants and young children (Carcillo Joseph et al. 2012).

Globally, efforts are underway to eliminate deficiencies in iron, zinc along with vitamin A, iodine, and folate. However, there are constraints in terms of access, affordability, and sustainability of these interventions. Therefore, we chose biofortification (increasing the minerals/vitamins in edible plant parts by genetic means) to improve the grain Fe and Zn concentration in staple crops. Here the intake is regular with no additional costs to the consumers.

Sorghum is a major food crop globally and it forms a principal staple for more than 500 million people in Sub-Saharan Africa and South Africa, which incidentally are the major food insecure, and micronutrient malnutrition-prone areas (Fig. 8.1). The idea behind biofortification research is to significantly increase the grain Fe and

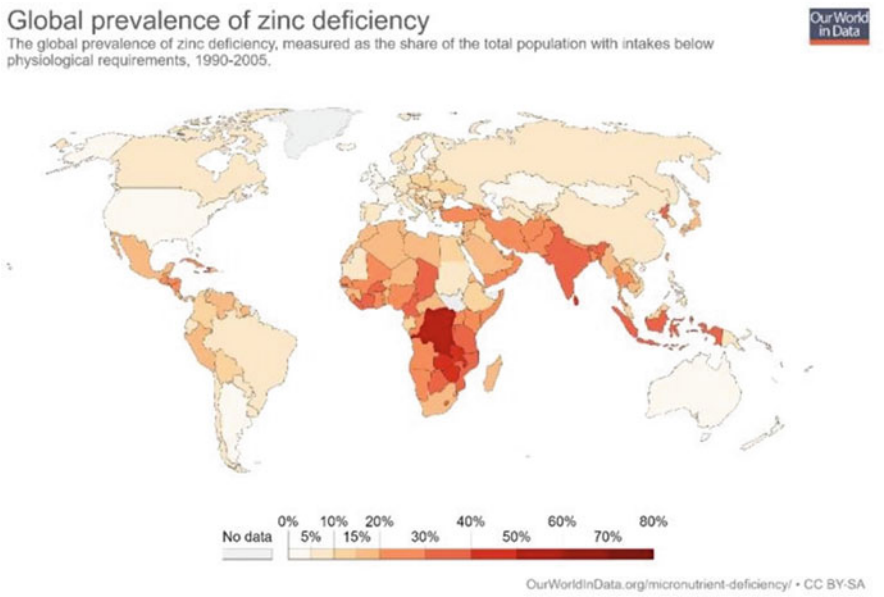

Fig. 8.1 Global prevalence of zinc deficiency (Wessels et al. 2013)

Zn concentration in the high yielding, farmer and market-preferred sorghum cultivars and delivering them into the food chain by increasing their adoption by the farmers through an innovative seed chain (Fig. 8.2). Here we are describing the success story of developing a novel biofortified cultivar "Parbhani Shakti" and an innovative seed chain built over years to increase its seed production and dissemination to farmers in Maharashtra state of India through a range of partnerships that include academia, Govt, public-funded seed agencies, and the farmers' groups.

2 Biofortifying Sorghum: Designing, Execution, and Product Development

To start with, we standardized the precise phenotyping methods for assessing the grain Fe and Zn in sorghum. The Inductively Coupled Plasma (ICP)—Optical Emission Spectrometry (OES) method standardized for assessing the germplasm, fixed breeding lines, and cultivars for Fe and Zn in sorghum. Also standardized the X-ray fluorescence spectrometer (XRF) for assessing the Fe and Zn, which is a low-cost, robust, and nondestructive method (Fig. 8.3). There is good correspondence between ICP and XRF methods for assessing the grain Fe and Zn but ICP is more accurate. So we used XRF for discarding the lines, segregating populations with low Fe and Zn and validate all high Fe and Zn lines with ICP method. To set up the baselines, the entire spectrum of sorghum cultivars (66) grown in India were assessed, and the Fe and Zn concentration in the most preferred cultivars was found

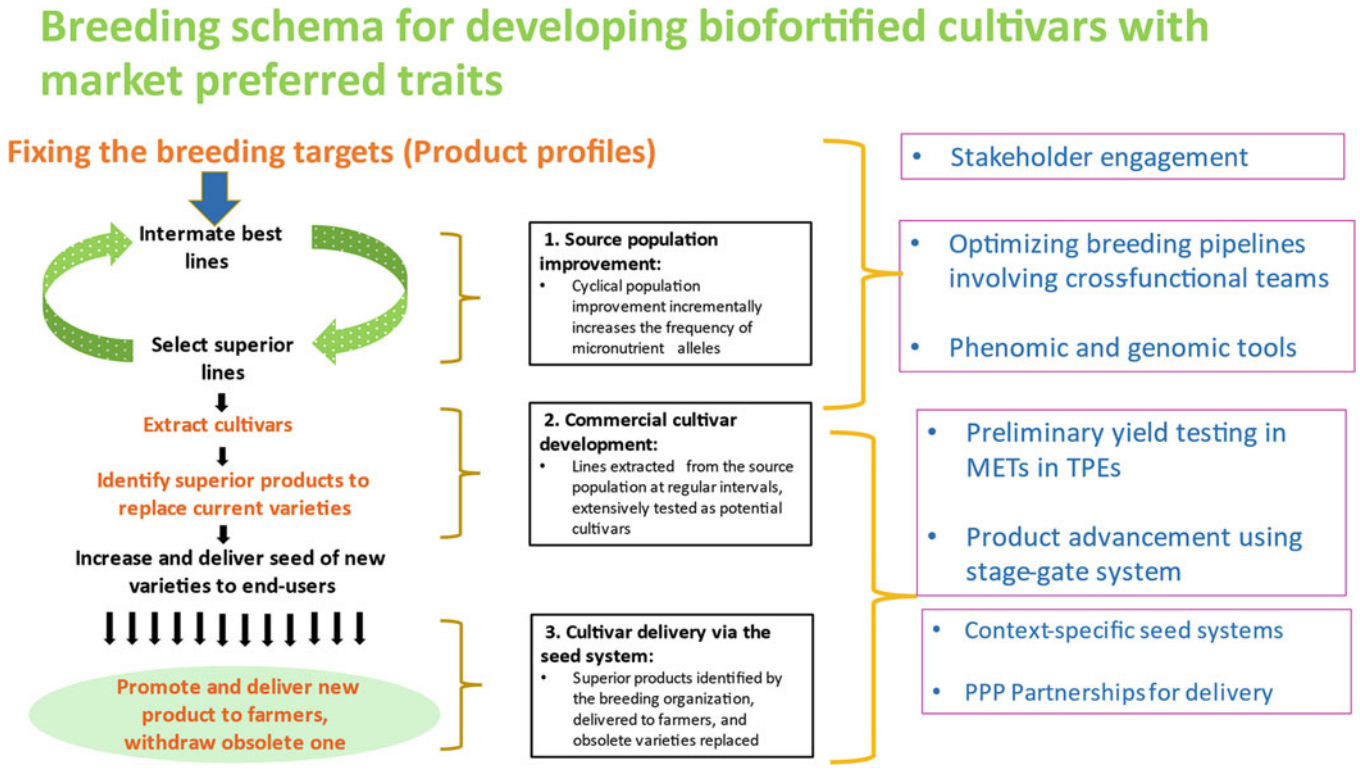

Fig. 8.2 Breeding scheme for developing biofortified sorghum cultivars with market-preferred traits. (Modified from Atlin et al. 2017)

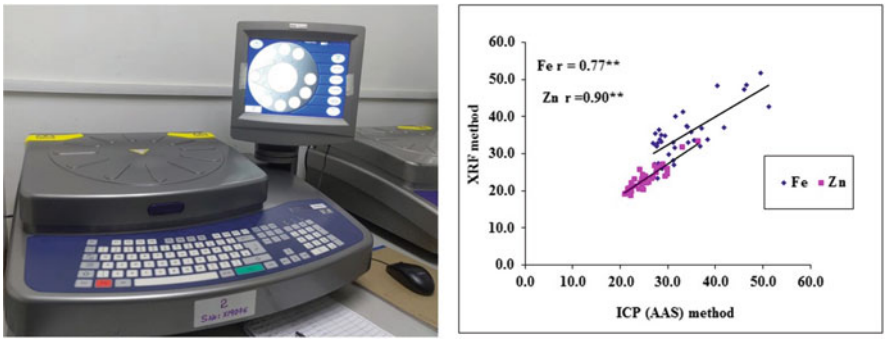

Fig. 8.3 XRF-low-cost, nondestructive, robust phenotyping technique for assessing Fe and Zn (Ashok Kumar et al. 2013)

to be low (30 ppm Fe and 20 ppm Zn), which were freezed as baselines for sorghum for increasing the grain Fe and Zn. We targeted to improve the Fe and Zn at least 50% higher than the baseline without compromising the grain yield, stover yield, and other preferred traits.

With the funding support received from HarvestPlus over several years, assessed the variability for grain Fe and Zn concentration in a large number of sorghum germplasm and breeding lines, parents; studied the gene action, trait associations, effect of micro and macronutrient fertilization on grain Fe and Zn; used micronutrients rich donors in crossing program and developed a strong breeding pipeline; developed a number of new hybrids (with selected parents for higher Zn and Fe), varieties and hybrid parents with high Zn and Fe concentration, which are under multilocation evaluation (Ashok Kumar et al. 2012, 2013, 2015; Phuke et al. 2017; Gaddameedi et al. 2020).

One of the improved varieties developed in the project, ICSR 14001, showed its yield superiority in multilocation on-farm testing in Maharashtra State of India and our partner Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani released it for commercial cultivation as "Parbhani Shakti" (Fig. 8.4). Besides high Fe (45 ppm) and Zn (32 ppm) it has higher protein content (11.9%) and low phytates (4.1 mg/100 g) means higher bioavailability of improved nutrients. Further, it is an excellent male parent for hybrids development and we developed more than 100 hybrids using it. Two more promising hybrids (ICSH 14001 and 14002) are under large-scale on-farm testing by the same partner in Maharashtra.

The release of first biofortified sorghum cultivar is a landmark towards addressing micronutrient malnutrition. However, it is important to ensure that farmers realize its benefits by farmers and the consumers adopt this variety. "Parbhani Shakti" being an OPV, private sector is less interested to multiply it. So, to enhance large-scale seed multiplication and dissemination of OPVs, an innovative "Seed Consortium" was developed by ICRISAT by bringing together all the major actors in seed chain to a common platform (Fig. 8.5).

Fig. 8.4 Improved sorghum variety (ICSR 14001) with high yield and higher grain Fe and Zn released as first biofortified sorghum variety "Parbhani Shakti" in India

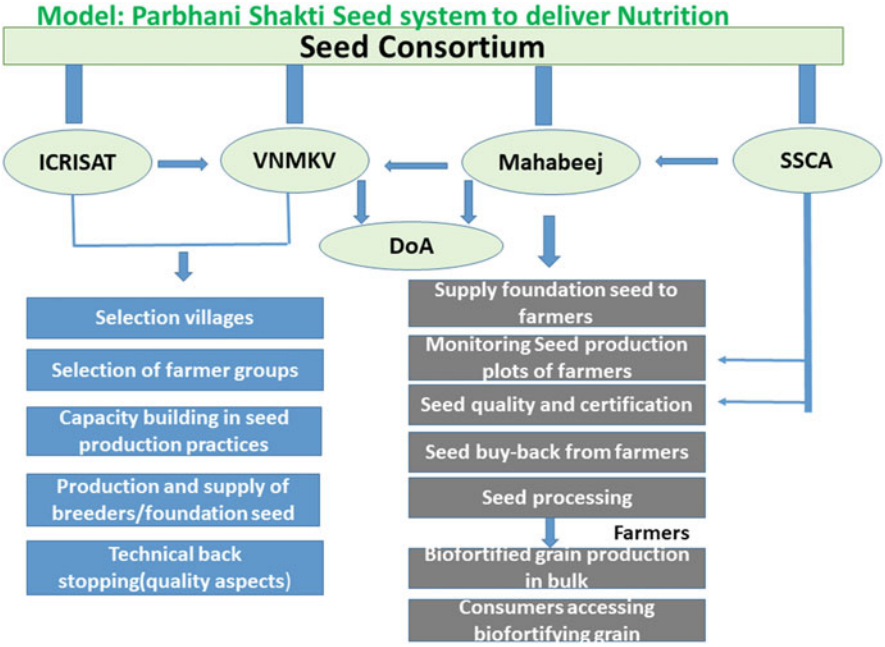

Fig. 8.5 Model seed system for biofortified sorghum variety "Parbhani Shakti"

3 Seed Consortium to Harness the Synergies and Product Delivery

The "Seed Consortium" was formed during 2013 involving the Indian NARS [ICAR—Indian Institute of Millets Research (IIMR), Mahatma Phule Krishi Vidyapeeth (MPKV), and Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV)], public sector seed agencies [Maharashtra State Seeds Corporation Ltd. (Mahabeej) and Maharashtra State Seeds Certification Agency (MSSCA)], Department of Agriculture, Govt of Maharashtra, and the seed farmers. Under this Consortium, in an annual meeting, all the partners come together, fix the targets to be achieved annually for the seed chain sustainability, and work together to achieve the targets on yearly basis. The State seeds corporation (Mahabeej) gives the buy-back guarantee and procure the seeds from the seed farmers. It processes the seeds and supplies to the farmers through its network in the entire country. With the concerted efforts by all the partners, the Seed Consortium made tremendous progress (Table 8.1). With this adoption of improved seeds coupled with increased adoption of management technologies, there is a steady increase in postrainy sorghum productivity in Maharashtra which is now more than 850 kg/ha and increasing. After the release of the first biofortified sorghum cultivar—Parbhani Shakti, Mahabeej came forward to mass multiply its seeds. In the 2018 postrainy season "Parbhani Shakti" was multiplied (50 tons) by both VNMKV and Mahabeej so as to reach maximum number of farmers in 2019 and this exercise is continued in 2019–20. By now the biofortified sorghum variety "Parbhani Shakti" is grown by 25,000 farmers and expanding. The biofortified grain is available in the market at the same price as normal sorghum grain and consumers get the benefit of higher micronutrients consumption without extra costs. As the intake is continuous (being staple crop) biofortified sorghum provides huge nutrition.

Once the improved cultivars developed and found highly suitable for scaling, to harness the synergies, the partners were identified in a way that all the partners in this endeavor have more or less similar goals—to improve the food and nutritional security of farmers, reduce the poverty and increase their incomes. However, not all partners were on the same page to readily come together. It needed demonstrations, joint field visits, farmers' explanations to convince "Mahabeej" to change its preferred cultivar for large-scale seed multiplication. While it had a strong preference for multiplication of M35-1, widely adapted landrace cultivar, the

Table 8.1 Sorghum seed production under the Seed Consortium initiative in Maharashtra

Year	Quantity of seed produced (tons)	No. of farmers supplied with improved variety seeds
2013	300	30,000
2014	1000	100,000
2015	1500	150,000
2016	2200	220,000
2017	2800	280,000
2018	3000	300,000

partnership could convince it to change its mindset by providing all the evidence, data, experiences and preferences of farmers, and finally the business opportunity in improved OPV seed multiplication. Then they agreed to give buy-back guarantee to the seed farmers. The perceived trade-offs by Mahabeej could be addressed by providing field evidences.

4 Development of Partnerships/Delivery Approaches

In this partnership, each partner had different competencies. Some are good in research (ICRISAT, ICAR-IIMR, VNMKV, MPKV), some have high capabilities in testing, release (MPKV, VNMKV); some are leaders in seed increase and commercialization (Mahabeej); and some in seed certification (MSSCA) and more importantly the farmers, as an integral part of this initiative. The Dept of Agriculture also played a key role in bringing the farmers together, in capacitating farmers in seed production.

5 Development of Metrics and Documentation

The group meets every year and fixes the targets for seed production with an aim to cover 10% of the total area in the state with improved seeds. The areas (geographies and extent) and farmers (numbers) for seed production are identified as per the targets fixed for the year. Each partner has designated roles and responsibilities and the entire activities are monitored from time to time. Farmers Rallies (4 Nos) are organized before crop harvest to ensure that more and more farmers (4×1500) are aware of these improved technologies and production packages and also to attract new farmers for seed production. At the end of the year, the progress made is reviewed, shortfalls addressed, and targets fixed for next year. And the cycle continues. New varieties are introduced into Seed Consortium as per the need.

The entire Seed Consortium initiative is well documented and policymakers are informed about the progress made. They are invited to the Annual Meeting of the "Seed Consortium" partners and their suggestions are taken for further improvement. The Govt of India is coming up with a "Millet Mission" to give a thrust for sorghum and millets production and both our University partners, VNMKV–Parbhani and MPKV–Rahuri, are identified as "Seed Hubs" in their Mission. Wide media coverage is given for all the on-farm activities to enhance the awareness and eventually the adoption rates.

6 Identifying Critical Partnerships

Multi-stakeholder partnerships with diverse competencies are very critical to carry forward the research and development initiatives. It is the first time in 40 years that ICRISAT worked with Mahabeej (one of the largest public sector seed production

bodies in the world) for large-scale seed production which helped the outreach, reaching 300,000 farmers per year and increasing every year. Developing mutual trust and respect and keeping all the partners on same page through continuous engagement is very critical. Sharing credit and engaging them from the beginning of variety release is the new approach we are following to have their buy-in from Day 1 of the new variety.

7 Lessons Learnt, Including Knowledge Gaps and Good Practices in Employing These Approaches at Scale

- Identification of right partners for the initiatives and convincing them of the need for the partnership
- Giving equal status to all partners and involving them from beginning
- Making every partner own the activities and apply themselves to achieve intended objectives
- Continuous engagement with partners for smooth flow of targeted activities
- Demonstrating the utility of initiatives by taking partners to farmers' fields
- Giving more credit to the partners upon achieving results
- Extensive coverage of activities and success stories in mainstream media.

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9 Pulse Crop Biofortification Toward Human Health, Targeting Prebiotic Carbohydrates, Protein, and Minerals

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Abstract

Recent data indicate that the world's food systems are unable to end hunger and all forms of malnutrition. In 2019, 690 million people were undernourished, and with the added impact of the COVID-19 pandemic, an additional ~130 million people may also become malnourished in 2020. Stunting and wasting result from poor nutrition in early childhood; however, malnutrition is rapidly transitioning into childhood obesity and overweight, especially in environments with high demand for processed foods. Food systems are failing to address food security, malnutrition, and diet-related noncommunicable diseases. New challenges, including pandemics, demographic changes, climate change, and globalization, are further adding to the complexity of the food system. The movement in crop production toward cereal monocultures and away from traditional food crops (pulses, tubers, roots) is linked to the malnutrition challenges facing many populations around the world today. Pulse crops have been a staple food in communities around the world for centuries. Pulses have high concentrations of protein (~30%) and prebiotic carbohydrates (10–15%), are low in fat (1–2%) and phytic acid, provide moderate energy, and are rich in iron (Fe), zinc (Zn), selenium (Se), folates, and carotenoids. Biofortification of pulse crops through conventional breeding and modern biotechnology to achieve target levels of

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nutrients is possible. The objective of this chapter is to discuss the promise of three major pulse crops (lentil, field pea, chickpea) in terms of nutritional breeding efforts and challenges of biofortification to improve human health and combat obesity and malnutrition.

Keywords

Nutritional breeding · Nutritional security · Prebiotic · Pulse biofortification

1 Introduction

The United Nations recently reported that more people are hungry than ever before. Some 690 million people were hungry in 2019, representing 8.9% of the world population. High food costs, low affordability, and inaccessibility of foods are the main reasons why many global communities cannot access healthy, nutritious whole foods daily. All forms of malnutrition are currently high in Asia but are expanding the fastest in Africa. Added complications due to the COVID-19 pandemic are increasing both chronic hunger and malnutrition, with more than 130 million additional people estimated to be affected by the end of 2020 (FAO 2020). Even without the pandemic, the trend of global malnutrition was anticipated to increase, meaning that 840 million people will be undernourished by 2030. This increasing trend in global food insecurity contributes to the low diet quality of women and children that leads to all forms of malnutrition. In 2019, 144 million (21.3%) children under the age of 5 were stunted, 47 million (6.9%) were wasted, 38.3 million (5.6%) were overweight, and 340 million were suffering from micronutrient malnutrition or "hidden hunger" (FAO 2020). Overcoming hunger and malnutrition requires a diet rich in essential nutrients and that is also low in cost and easily accessible. Fresh vegetables, fruits, and animal protein are the food groups that are typically out of reach in terms of affordability for most vulnerable populations. Therefore, the transformation of food systems should focus on reducing the cost of healthy foods and increasing accessibility. Government policies should invest in smallholder farmers to grow sustainable staple food crops, especially pulse crops that can provide all essential nutrients for a low price.

Feeding an anticipated global population of ten billion people by 2050 while protecting the environment is a significant challenge related to global food security (Reganold and Wachter 2016). The ecological and nutritional benefits of pulse crops have been well known for centuries, but little evidence is available with respect to how to redesign global food systems with diverse pulse crops by making this option more attractive to smallholder farmers and consumers (Reckling et al. 2020). Pulses provide ecological, economic, and social benefits via biological N fixation, enhanced biodiversity, and the end goal of healthy food systems toward combating malnutrition. During the green revolution, calorie-focused crop production replaced traditional pulse crops that provided micronutrients to many people in Asia and Africa (Bouis and Welch 2010). The global population continues to increase, with more

than 90 million additional mouths to feed each year, meaning global food demand is expected to double by 2050. With limited arable lands, decreasing soil fertility, climate change, and declining water resources, current food systems cannot provide sufficient nutrients to the global population. Dependence on animal products for daily nutrients is not an option for most communities and is becoming even more difficult in developed countries. Therefore, traditional pulse crops need to be investigated in terms of their ability to provide better food and nutrition solutions toward improved human health. The objective of this chapter is to discuss the nutritional promise of three major pulse crops and their links to human health, nutritional breeding efforts, and challenges of biofortification with the aim to combat malnutrition on a global scale.

1.1 Agriculture and Nutrition

A large number of global communities are facing the double burden of malnutrition, i.e., undernutrition (micronutrient malnutrition, stunting, wasting, and underweight) concurrent with obesity and diet-related noncommunicable diseases. Over 30% of the global population (two billion people) suffer from micronutrient malnutrition, 2.28 billion people are overweight, and 150 million children are stunted (WHO 2020). More people are obese and overweight than underweight; this is a global phenomenon with the exception of parts of sub-Saharan Africa and Asia. Obesogenic environments are increasing, while the causes of micronutrient malnutrition persist as a result of the failing food system. The double burden of malnutrition is a result of the rapid transitioning of global food systems that increased the consumption of ultra-processed foods that are high in sugar and fat (Popkin et al. 2020).

Staple food crop production has a major impact on food systems. Micronutrient malnutrition is a result of decreasing the production of micronutrient-rich staple food crops such as pulses (lentils, field peas, chickpea, and common beans; Fig. 9.1). In recent years, smallholder farmers have typically adopted simple rotations with high-yielding cash crops, i.e., wheat, maize, and rice, and abandoned traditional low-calorie pulse crops that are rich in protein, low digestible carbohydrates, and micronutrients. This trend has created unforeseen consequences over the years by reducing micronutrient-rich food crops in farming systems that previously depended on more diverse crops (which are no longer part of food systems) (Fig. 9.1). Nutritional transitions are increasing the rates of the double burden of malnutrition, where societies are shifting from traditional to calorie-rich diets derived from newly adopted unhealthy food systems (Bouis and Welch 2010; Thavarajah et al. 2014).

Agriculture is the primary food source of micronutrients for the human diet, so agricultural systems must be contributing to the problem as cereal and sugar crops do not provide adequate human nutritional needs. This leads to the fundamental question: *how can agriculture adapt to provide nutritional security for the world's growing population?* A holistic food system approach is essential to link human nutritional needs with staple food production to fulfill food and nutritional security

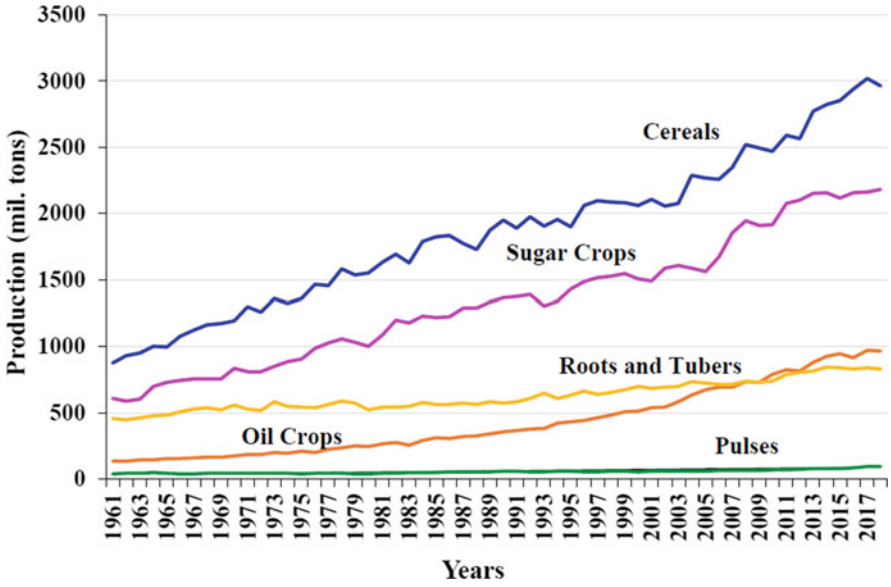

Fig. 9.1 Staple food crop production change from 1961 to 2017 (FAOSTATS 2020)

needs. Agricultural research in the areas of plant breeding, biotechnology, and agronomy should focus on improving the nutritional quality of these staple food crops, while public and government sectors should ensure the agriculture interventions will be sustainable as well as adopted by farmers and consumers. Agricultural interventions should also be used as a primary tool to fight the double burden of malnutrition.

Advances in staple food production occurred during the "green revolution," when the food supply of cereal crops (e.g., rice, wheat, maize) was the focus (Fig. 9.1). These cereals are an excellent source of carbohydrates but contain only a small amount of protein and few micronutrients. The movement in crop production toward cereal monocultures and away from traditional food crops (pulses, tubers, roots) is linked to the malnutrition challenges facing many populations around the world today (Bouis and Welch 2010; Thavarajah et al. 2014; Popkin et al. 2020). Nutritional transitions are also contributing to malnutrition, as societies are switching from traditional plant-based diets to more caloric, high sugar and fat diets derived from adopting developed nations' food systems (Wells et al. 2020). Several approaches can be taken to increase nutrient output from staple food crops, such as (1) field selection for appropriate soil nutrients, (2) agronomic techniques including micronutrient fertilizer application and diversified cropping systems with crop rotations, and (3) biofortification: increasing nutrient concentration and bioavailability in the edible seed using modern plant breeding, genetic, and biotechnology tools. These edible seeds also contain antinutritional substances that may influence nutrient bioavailability to humans. Therefore, nutritional breeding is

complex and must consider nutrients and their bioavailability before advancing genotypes in breeding programs.

1.2 Biofortification

Biofortification using conventional plant breeding and genomic tools is an effective agricultural intervention to alleviate global micronutrient malnutrition. The CGIAR HarvestPlus program was established in 2003, and, since then, 150 biofortified varieties of 10 crops have been released in 30 countries throughout Asia, Africa, and South America (Bouis and Saltzman 2017). More than 20 million smallholder farmers are now growing these biofortified varieties; however, to meet the goal of reaching one billion farmers by 2030, three key barriers must be overcome: (1) integration of biofortified traits into public plant breeding programs, (2) continued consumer demand for biofortified foods, and (3) increased and ongoing support from the public and private policies, programs, and investments. These programs will require building new and expanding current partnerships, ongoing government funding streams, and increasing partner capacity. HarvestPlus has been successfully delivering biofortification tools to vulnerable populations around the world to improve their crop productivity as well as combat micronutrient malnutrition (Bouis and Welch 2010; Bouis and Saltzman 2017). Pulse crops have been studied for mineral biofortification in many breeding programs around the world, especially for iron (Fe), zinc (Zn), and selenium (Se) (Thavarajah et al. 2008, 2009a, b, 2015a, b). Other nutritional traits being targeted include prebiotic carbohydrates, protein quality, carotenoids, folates, antioxidants, and bioactive compounds. However, as a result of the quantitative genetic nature of these nutritional traits as well as the chemical phenotyping barrier, more in-depth research is required to integrate these traits into local breeding programs.

2 Global Pulse Production

In 2018, world pulse production was 92.3 million metric tons (FAOSTATS 2020). This represented an increase of 36 million metric tons (63%) over the previous 20 years (1998–2018) as a result of production increases in North America, sub-Saharan Africa, South Asia, Latin America, and the Caribbean. In particular, higher production has been reported for chickpea (+8.3 million metric tons), lentil (+3.6 million metric tons), and field pea (+1.2 million metric tons) over these two decades. Pulse production increased by 17.3 million metric tons in Asia (with South Asia contributing 12 million metric tons), 10.5 million metric tons in sub-Saharan Africa, 4.8 million metric tons in North America, and 2.2 million metric tons in Latin America and the Caribbean.

The key driver of the pulse production increase over these two decades was global research collaboration among the CGIAR institutes, including the International Center for Agricultural Research in the Dry Areas (ICARDA), International Crops

Research Institute for the Semi-Arid Tropics (ICRISAT), International Center for Tropical Agriculture (CIAT), and universities around the world. These CGIAR institutes now hold significant pulse germplasm collections. For example, ICRISAT has 20,764 accessions of chickpea; ICARDA has 11,877 accessions of lentil; and CIAT holds 37,938 accessions of Phaseolus bean. National gene banks around the world, including the USDA-ARS, hold substantial repositories of genetic resources for pulse crops; for example, the national gene banks in India have over 63,000 accessions of different pulse crops. The establishment of the Commission on Genetic Resources for Food and Agriculture in the Food and Agriculture Organization of the United Nations (FAO) and the International Treaty on Plant Genetic Resources for Food and Agriculture (PGRFA) in 2001 has supported the enhancement of genetic repositories and new cultivar development around the world (FAO 2020). Today, significant global pulse production takes place on smallholder farms. Additionally, the prairie provinces of Canada (Saskatchewan, Alberta, and Manitoba), southeastern and western Australia, and the Midwest region of the USA have adopted lentil, field pea, and chickpea into their cropping systems. As a result of suitable environmental conditions and producer interest in new cash winter crops, pulse crops are also becoming popular in the southern United States (e.g., South Carolina, Georgia, North Carolina) (Pulse Pod 2020).

2.1 Pulse Protein

Plant proteins provide more than two-thirds of daily protein requirements to world populations; this contribution to the US population is around one third. The need for plant protein will continue to grow concurrent with increasing demand and limited resources for animal protein production, relatively low environmental burden associated with plant proteins, and the shift of consumer preferences. However, challenges related to plant proteins must be overcome to provide a nutritious and safe protein choice. Proteins are the only source of essential and non-essential amino acids for various human bodily functions. Of the 21 different amino acids, 9 (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) are crucial because the human body cannot synthesize them. Not all plant protein sources are equal or sources of all essential amino acids. The absence of any one or more of these essential amino acids lowers the nutritional quality of a protein source; this can have negative impacts on the health of consumers dependent on these sources. Human diets from pulses are generally low in essential methionine and conditionally essential cysteine. Cereal proteins are usually rich in both of these amino acids. Therefore, a variety of plant protein sources will ensure all essential amino acids for improved human health.

Globally, the plant-based protein market is expected to increase to a $9.5 billion industry by 2025. North America is the largest region contributing to increasing demand, and, in 2018, representing 38.6% of the global protein market. Plant-based proteins are an inexpensive choice for many populations and a vital source of daily essential amino acids, especially in Asia and Africa. The global demand for

plant-based protein has shifted; the North American market is significant due to increased awareness of ecosystem health, climate change, animal welfare, and antibiotic resistance. These concerns are putting the spotlight on plant-based proteins, and new food choices available to Americans include items such as the "Beyond Burger" and "Impossible Burger." However, the nutritional aspects of plant-based burgers have met with criticisms: (1) they contain processed soy as the only protein; (2) salt and saturated fat content are higher than regular beef burgers; and (3) they lack a whole food perspective (e.g., dietary fiber, vitamins, minerals). In fact, plant-based proteins have several limitations in terms of human nutritional benefit: (1) most lack one or more essential amino acids (e.g., sulfur-containing amino acids); (2) they are often not fully digestible or bioavailable as a human food or animal feed (e.g., protease inhibitors); (3) changes in protein structure, stability, and function occur during thermal processing (e.g., lack of emulsion stabilities); and (4) plant chemicals, toxins, and pesticides are concentrated during protein extraction and drying (e.g., they are part of protein structures and not removed during protein isolation). Although soy protein is the most abundantly used protein today, evidence in terms of its health implications is conflicting. Soy protein isolates have antinutritional, allergenic, and hormone-mimicking compounds (e.g., phytoestrogens). Therefore, pursuing nutritional breeding or "biofortification" of other traditional pulse crops is vital to overcome current issues related to plant-based food applications.

The majority (70–80%) of pulse proteins, especially those from field pea, are globulins while the remainder is albumins. The globulin protein legumin (11S, 300–400 kDa) is a hexameric homo-oligomer with subunits of approximately 60 kDa. However, the globulin protein vicilin (7S, 15–18 kDa) is trimeric, with a main subunit of 50 kDa. Convicilin is a minor globulin that is a trimer or tetramer of 71 kDa subunits. In addition to globulins and albumins, pulse proteins have minor prolamin and glutamine protein components. The majority of current plant protein isolation methods involve alkaline/acid conditions, high-pressure filter separations, and temperature treatments. Depending on the raw material plant source, expected protein yields, and economic incentives, the protein isolation conditions applied can be so harsh as to denature the native protein structure. The long-term sustainability and impacts on human health of a plant protein source are related to its ability to provide a balanced supply of all amino acids in a highly digestible form.

2.2 Pulses and Human Health

Practices favoring cereals over pulses may have compromised human health, as deficiencies in Fe, Zn, iodine (I), Se, and vitamin A continue to be observed globally (Thavarajah et al. 2014). Therefore, nutritional breeding and biofortification of pulses to increase human health are of interest. Over the last 10,000 years, humans evolved within an ecosystem in which pulse crops constituted an integral part of a typical diet (Purugganan and Fuller 2009). Pulses were consumed as a staple food in most historical contexts and remain in many populations worldwide, eaten in large

amounts throughout the day, and generally as the primary source of calories and protein. Not surprisingly, many health benefits are associated with pulse-rich diets and have been demonstrated in both observational studies and randomized controlled trials (RCTs) (Viguiliouk et al. 2019; Papandreou et al. 2019; Ferreira et al. 2020). Observational studies of over 200,000 individuals suggest legume intake is associated with a modest all-cause mortality survival benefit (relative risk [RR], 0.93; 95% confidence interval [95% CI], 0.87–0.99) (Li et al. 2017). Pulse intake (highest intake compared with the lowest intake) is further associated with decreased incidence of cardiovascular disease (RR, 0.92; 95% CI, 0.85–0.99), coronary heart disease (RR, 0.90; 95% CI, 0.83–0.99), hypertension (RR, 0.91; 95% CI, 0.86–0.97), and obesity (RR, 0.87; 95% CI, 0.81–0.94) (Viguiliouk et al. 2019). In an observational study of over 7200 participants, the highest pulse consumption tertile, compared with the lowest tertile, was associated with a lower risk of cancer mortality (hazard ratios [HR], 0.51; 95% CI, 0.31–0.84; $P = 0.009$) (Papandreou et al. 2019). Primary effects identified in RCTs are improvements in metabolic measures (e.g., serum lipid profiles, glucose, fasting insulin, hemoglobin A1c, insulin resistance), blood pressure, anthropometric measures (e.g., body weight, waist circumference), inflammatory biomarkers, oxidative stress, and fecal pathogenic bacteria (Ferreira et al. 2020). Endpoints, such as mortality, cancer incidence, or cardiovascular disease, require very large study populations followed over long durations, and such studies simply have not been performed.

Bacteria in the human digestive system that ferment prebiotic carbohydrates are associated with several health benefits, including reduced obesity (Schwiertz et al. 2010) and insulin dependence (Gao et al. 2009), as well as protection against the development of colorectal cancer (Keku et al. 2015). A hypothesis with growing support has been put forth that asserts the benefits from dietary pulses are, in part, moderated through interactions in the hindgut microbiome (Marinangeli et al. 2020). Pulses contain relatively large fractions of carbohydrates, broadly categorized under dietary fibers, which have known prebiotic functions (Lockyer and Stanner 2019). These prebiotic fibers have been associated with health benefits in humans, thus forming the basis for this hypothesis. However, large clinical trials to further evaluate this idea are warranted. Importantly, the quality of evidence in the majority of these studies is low, owing largely to the many difficulties associated with conducting large-scale dietary studies. The human diet is vastly complex, and our global understanding thereof is grossly limited (Barabási et al. 2020). Robust, systematic investigations of dietary composition and human health are urgently needed. These should ideally focus on the staple food crops that feed large segments of the global population and are already known to provide health benefits, such as pulses (lentil, field pea, and chickpea).

3 Lentil

The Leguminosae family has 800 genera and 20,000 species. Among these species, lentil (*L. culinaris* L.) is a self-pollinated diploid cool-season legume species with seven chromosome pairs and a relatively large genome of ~4 Gbp (Polanco et al. 2018). Studies by ICARDA lentil breeders report considerable lentil genetic diversity for agro-morphological and phenological characteristics in the ICARDA lentil core collection. Several molecular markers have been developed which characterize lentil genetic resources for disease resistance, drought tolerance, heat tolerance, phenology, and plant morphology (Erskine and Choudhary 1986; Khazaei et al. 2016). As a result of the recent availability of the draft lentil genome, researchers are now able to use genome-wide association mapping for complex genetic traits.

Lentil, or *poor man's meat*, is a whole food that is low in fat and high in protein, prebiotic carbohydrates, and a range of vitamins and minerals. A 50-g serving provides 3.7–4.5 mg of Fe, 2.2–2.7 mg of Zn, and 22–34 μg of Se and is very low in phytic acid (2.5–4.4 mg/g) and protein inhibitors (Thavarajah et al. 2008, 2009a, b). Lentil has a short cooking time (10–12 min) and low processing requirements (dehulling only). Mineral biofortification has successfully been adopted into lentil breeding programs around the world. Mineral biofortification depends on the food matrix factors that govern the absolute bioavailability to humans. Our model is to first screen the Fe bioavailability in germplasm based on food matrix factors, including Fe bioavailability promoters (ascorbic acid, prebiotic carbohydrates, phytoferritin, and carotenoids) and inhibitors (phytic acid, kaempferol, gallic acid, chlorogenic acid). Once appropriate breeding efforts have been carried out based on the phenotyping data, Fe bioavailability studies are carried out using the in vitro Caco-2 cell model or mouse model using only the selected advanced breeding lines. Finally, these selected varieties are used in human trials to test the true Fe bioavailability of lentils (Thavarajah et al. 2011a, b, 2015a, b).

Recently, the ICARDA lentil breeding program is released between 10 and 30 Fe- and Zn-biofortified lentil cultivars for Africa and Asia following the screening of a germplasm collection of >1500 lentil accessions, including landraces, wild types, and breeding lines. The new lentil cultivars had seed concentrations of 43–132 mg/kg Fe and 22–78 mg/kg Zn; a subsequent study reported 41–109 mg/kg for Fe and 22–78 mg/kg for Zn (Baum et al. 2008). Numerous high Fe and/or Zn lentil genotypes have been released: "ILL 5883" (73 mg/kg Fe) and "ILL 6994" (72 mg/kg Fe) in Syria; "ILL 7711" (74 mg/kg Fe) in Portugal; "Alemaya" (82 mg/kg Fe, 66 mg/kg Zn) in Ethiopia; "Meyveci-2001" (53 mg/kg of Zn) in Turkey; and "Sisir" (98 mg/kg Fe, 64 mg/kg Zn), "Khajurah-2" (94 mg/kg Fe, 54 mg/kg Zn), "Barimasur-5" (86 mg/kg Fe, 59 mg/kg Zn), and "Barimasur-6" (86 mg/kg Fe, 63 mg/kg Zn) in Nepal (Baum et al. 2008; Erskine and Choudhary 1986; Kumar et al. 2015). A recent study from Nepal reported lentil Fe concentrations ranging from 72 to 154 mg/kg and Zn concentrations ranging from 54 to 70 mg/kg (Darai et al. 2020). Fe- and Zn-biofortified lentil cultivars are now available in India, Bangladesh, Nepal, Morocco, and Ethiopia. However, marker-assisted breeding is

not yet available to these breeding programs (Kumar et al. 2015; Khazaei et al. 2016, 2017).

Selenium (Se) is an equally essential element to humans as Fe and Zn. Plants uptake Se from the soil and translocate it to the chloroplast, where it follows sulfur (S) and/or phosphorus (P) assimilation pathways. Lentils grown in aqueous solutions containing 2 mg/L Se show increased germination percentage, appear healthier compared to control plants, and have seed total Se concentrations of 2.5–8.7 mg/kg. Further, significant correlations are evident between Se fertilization and grain yield, biomass, root volume, nodule number, and seed Se concentration in lentil grown under controlled conditions (Thavarajah, unpublished data). A correlation analysis was performed to evaluate the link between seed Se level and lentil grain yield data from two lentil breeding programs: (1) lentil grown in high Se soils in Canada ($n = 912$; mean lentil yield = 1032 kg/ha and seed Se = 1445 μg/kg) and (2) lentil grown in low Se soils in Nepal ($n = 255$; mean lentil yield = 618 kg/ha, and seed Se = 180 μg/kg). The correlation was significant and positive (0.71) for lentils grown at different locations; that is, lentil yields are proportional to Se availability in the soils (Thavarajah et al. 2008, 2011a).

Soil Se levels govern lentil germination and plant health. A germination study was conducted with two Se treatments [0 (control) and 30 kg of Se/ha] with three replicates to determine how low-dose Se fertilizer application at germination affects lentil seedling biomass, antioxidant activity, and Se uptake of 26 cultivated lentil accessions from the USDA (Thavarajah et al. 2017). Se treatment significantly increased seedling biomass in lentil genotypes vs. the control. Similarly, relative biomass increased; specifically, PI320937, PI533690, PI518732, W627767, W627754, and PI533693 demonstrated biomass increases of more than 50% compared to their controls. Among these genotypes, PI533690 and PI533693 showed a >100% biomass increase compared to controls. As expected, Se treatment (30 kg of Se/ha) significantly increased seedling Se concentration for all genotypes; PI320937 showed the highest Se uptake (6.2 μg/g) and W627780 (1.1 μg/g) the lowest (Thavarajah et al. 2017). A separate field study quantified the seed Se concentration of 191 ICARDA lentil wild accessions grown in Terbol, Lebanon, without the addition of Se fertilizer. Seed Se concentrations of these wild accessions ranged from 0 to 2.5 μg/g; accessions originating from Syria (0–2.5 μg/g) and Turkey (0–2.4 μg/g) had the highest seed Se. Frequency distribution analysis revealed that seed Se for 63% of accessions was between 0.25 and 0.75 μg/g. Thus, a single 50-g serving of lentil has the potential to provide adequate dietary Se [20–60% of recommended daily allowance (RDA)]. Incorporating a diverse panel of wild lentil germplasm into Se biofortification programs will increase genetic diversity for effective genetic mapping for improved lentil seed Se nutrition and plant productivity (Ekanayake et al. 2015, 2017; Thavarajah et al. 2017).

Lentil is an excellent source of prebiotic carbohydrates and supports a healthy gut microbiome. Naturally occurring lentil prebiotic carbohydrates can be divided into two major groups: dietary fiber and sugar alcohols (SAs). Dietary fiber includes starch polysaccharides [resistant starch (RS)] and non-starch polysaccharides [raffinose family oligosaccharides (RFOs) and fructooligosaccharides (FOSs)]. A cup of

lentil provides 13–15 g of prebiotic carbohydrates; this amount doubles after cooling and reheating (Johnson et al. 2013, 2015). Further, lentil provides >80% of the suggested RDA of prebiotic carbohydrates, which are associated with reduced weight gain via modulating the human gut microbiome. A recent study indicates rats fed a lentil diet had significantly lower mean body weight (443 g/rat) than those fed control (511 g/rat) or corn starch (502 g/rat) diets. Further, the mean percent body fat and body plasma triacylglycerol (TG) concentration were lower, and lean body mass was higher, in rats fed the lentil vs. the corn diet. The fecal abundance of healthy bacteria, Actinobacteria and Bacteroidetes, increased in rats fed the lentil diet, while the abundance of Firmicutes (a bacterial phylum comprised of multiple pathogenic species) decreased (Siva et al. 2018). Therefore, lentil is promising as a plant-based food to reduce obesity-related noncommunicable diseases, which are a rising health concern in the USA.

4 Field Pea

Field pea (*Pisum sativum* L.) is an attractive, alternative staple crop to complement cereals, as it has higher amounts of protein, prebiotic carbohydrates, and fiber (Kumar and Pandey 2020). It is a member of the tribe Fabeae, family Leguminosae, subfamily Papilionoidaea which has two distinct clades: Hologalegina that first evolved 50 million years ago, followed by Phaseolid that evolved 45 million years ago (Foyer et al. 2016). Field pea was one of the first domesticated crops, originating from the Middle East (Syria, Iraq, and Iran) ~10,000 years ago. The pea genome is seven chromosomes, with five acrocentric (3, 4, 5, 6, 7) and two sub-metacentric chromosomes (1, 2). Pea has a large, diploid genome size, at 4.45 Gb, and is mostly made up of repetitive sequences, constituting 76–97% of the genome (Kreplak et al. 2019). Despite its superior nutritional quality, cultivated land acreage for field pea and other pulse crops has decreased over the past 30 years with no net gain of field pea production as yields have increased (Stagnari et al. 2017; Foyer et al. 2016; Powers and Thavarajah 2019). Additionally, global markets and policies for field pea are limited and unfavorable, so crop improvement has lagged in comparison to cereals, keeping field pea yields small and unpredictable (Foyer et al. 2016; Stagnari et al. 2017).

Field pea is highly nutritious (Table 9.1), and the protein concentrations range from 21.2 to 32.9%, total carbohydrate concentrations range from 56.6 to 78.6%, and starch and crude fiber concentrations are high (45 and 68%, respectively) (Kumar and Pandey 2020). Field pea has low-fat concentration (0.8–6.1%) and a low glycemic index, both of which support a healthy diet and can help alleviate diet-related illnesses such as type II diabetes (Marinangeli et al. 2009; Kumar and Pandey 2020). Field pea is naturally rich in Fe, Zn, and magnesium (Mg), providing between 28–68%, 36–78%, and 34–46% of the RDA of these micronutrients, respectively (Amarakoon et al. 2012). Thavarajah et al. (2010) found that field pea grown in Saskatchewan, Canada, is naturally enriched (373–519 μg/kg) in Se and can provide 68–94% of the RDA for Se. Similar to lentil, field pea is also low in phytic acid

Table 9.1 Nutrient composition of pulses: lentil, field pea, and chickpea

Nutrient (per 100 g serving)	Lentil	Field pea	Chickpea
Water (g)	8.3	8.7	7.7
Energy (kcal)	352	364	378
Protein (g)	24.6	23.1	20.5
Total lipid (g)	1.1	3.9	6.0
Ash (g)	2.7	2.7	2.7
Carbohydrate, by difference (g)	63.4	61.6	62.9
Fiber, total dietary (g)	10.7	22.2	12.2
Sugars (g)	2.0	3.1	10.7
Minerals (mg)			
Calcium (Ca)	35	46	57
Iron (Fe)	6.5	4.7	4.3
Magnesium (Mg)	47	63	79
Phosphorus (P)	281	334	252
Potassium (K)	677	852	718
Sodium (Na)	6	5	24
Zinc (Zn)	3.3	3.5	2.8
Copper (Cu)	0.75	0.81	0.66
Manganese (Mn)	1.4	1.2	21.3
Selenium (Se) (μg)	0.1	10.7	0
Vitamins			
Vitamin C (mg)	4.4	1.8	4
Thiamin (mg)	0.87	0.72	0.48
Riboflavin (mg)	0.21	0.24	0.21
Niacin (mg)	2.6	3.61	1.54
Vitamin B-6 (mg)	0.54	0.14	0.54
Folate, dietary folate equivalent (μg)	479	15	557
Vitamin A, retinol activity equivalents (μg)	2	7	3
Vitamin E (mg)	0.49	0.12	0
Vitamin K (μg)	5	15.9	9

Source: Original data obtained from the USDA Nutrient Database for Standard Reference (https://fdc.nal.usda.gov/fdc-app.html#/food-details/172420/nutrients)

(4.9–7.1 mg/g) (Amarakoon et al. 2012). Field pea contains significant concentrations of Fe bioavailability promoters: xanthophyll (17 mg/100 g), cantha-xanthin (68 mg/100 g), β-carotene (680 μg/100 g), kestose (1433 mg/100 g), quer-cetin (51.7 mg/100 g), and ferulic acid (56.1 mg/100 g). The phytic acid concentration of field peas is naturally low (2.7–3.2 mg/g), and the phytic acid:Fe molar ratio ranges between 5.0 and 5.6. Cultivars "CDC Golden" and "DS Admiral" were selected to develop mapping populations for Fe biofortification studies (Amarakoon et al. 2012, 2015). Field pea is a good food source of Fe and Zn, and the selection of genetic material to enrich micronutrients in conjunction with the growing location may further enhance mineral concentrations.

Biofortification strategies for field pea consist of two approaches: foliar (fertilizer) application and genetic improvement through traditional breeding. A study of Zn biofortification of field pea demonstrates that spraying a Zn fertilizer onto the soil and leaves of growing field pea increases the grain concentration of Zn above 60 mg/kg, which would provide more than 80% of the RDA (Poblaciones and Rengel 2016). These researchers conducted a similar experiment with the foliar application of both Zn and Se. The combined application of both elements can provide approximately 50% of the RDA for Zn and 45% of the RDA for Se (Poblaciones and Rengel 2017). However, given that much of the world's farmland is managed by small (12%) or family-operated farmers (75%), the availability and affordability of foliar nutrient sprays may hinder the success and sustainability of this biofortification approach (Lowder et al. 2016).

A lack of genomic resources partially explains the minimal biofortification studies in field pea, but the reference genome has recently been released, which will aid in increasing research (Kreplak et al. 2019). Several quantitative trait loci (QTL) mapping and genome-wide association studies have identified QTLs and single nucleotide polymorphism (SNP) markers significantly associated with Zn, Fe, Se, and folate content in field pea. Large variation for Fe concentration exists across field pea germplasm (Jha and Warkentin 2020). Diapari et al. (2015) and Dissanayaka (2019) found a total of 12 SNPs associated with Fe concentration across 94 and 177 field pea accessions, respectively. Ma et al. (2017) found five QTLs, and Gali et al. (2018) also identified several QTLs associated with Fe concentration across four individual genotypes. Grain Zn concentration is also diverse in field pea; nine SNP markers and multiple QTLs across different field pea populations were found to have significant associations with grain Zn content (Ma et al. 2017; Gali et al. 2018; Dissanayaka 2019; Diapari et al. 2015). As previously stated, field pea is relatively high in Se (Thavarajah et al. 2010), with multiple QTLs and 44 SNPs reported to be associated with Se concentration. However, environmental effects and soil Se concentration are likely to play a larger role in grain Se accumulation than genotype (Gali et al. 2018; Dissanayaka 2019; Jha and Warkentin 2020). Recent work by Jha et al. (2020) identified 5 SNPs associated with total folates, 15 SNPs for 5-methyltetrahydrofolate, 8 SNPs for 5-formyltetrahydrofolate, and 3 SNPs for tetrahydrofolate. Carotenoids in field pea accessions have also been reported, with some accessions having greater total carotenoid (5.8–26.9 μg/g) and β-carotene (2.6 μg/g) concentrations than biofortified "Golden Rice" (1.6 μg/g) (Holasova et al. 2009; Ashokkumar et al. 2014, 2015; Beyer et al. 2002; Jha and Warkentin 2020). As genomic technology and genetic research in field pea improve, more markers for different micronutrients will be discovered and implemented into breeding programs.

5 Chickpea

Chickpea is one of the eight "founder crops" domesticated by Neolithic societies 8000–12,000 years ago (Zohary et al. 2012). Domesticated chickpea (*Cicer arientinum* L.) is thought to have been derived from initial selections in the progenitor species *C. reticulatum* Ladizinsky (Ladizinsky and Alder 1976), which had a limited distribution throughout southern Turkey. Chickpea is currently the third most important pulse crop of global production, after drybean (*Phaseolus vulgaris* L.) and field pea, with >17.1 million MT produced in 2018 (FAOSTATS 2020). India is responsible for more than 80% of annual global production, with Myanmar, Ethiopia, Turkey, and Pakistan being other major producers (FAOSTATS 2020). Chickpea has two major classes: the macrosperma, or "kabuli" class, and the microsperma, or "desi" class (Toker 2009). Kabuli chickpea seeds are shaped like an "owl head" and are larger and lighter in color than desi-type chickpeas, which have a "teardrop" seed shape. Kabuli-type chickpeas are cooked then used for salads, canned, and eaten whole or used to make hummus, while desi chickpeas are predominately split and then cooked (Newman et al. 1998).

Research on the biofortification of chickpea has largely focused on characterizing the nutritional qualities of adapted breeding lines and cultivars. Seed concentrations have been determined for minerals (Bueckert et al. 2011; Ray et al. 2014; Vandemark et al. 2018), dietary fiber (Chen et al. 2016), and prebiotic carbohydrates (Vandemark et al. 2019). Selenium has consistently been shown to be the least abundant mineral in chickpea seed, ranging in concentrations from 33 to 73 μg/100 g, while K (potassium) is the most abundant, with concentrations greater than 10,000 μg/100 g (Thavarajah and Thavarajah 2012a, b; Ray et al. 2014; Vandemark et al. 2018). Estimates of Fe concentration in chickpea breeding lines and cultivars grown in North America range from 5.2 mg/100 g (Vandemark et al. 2018) to 6.0 mg/100 g (Thavarajah and Thavarajah 2012a, b). Estimates of Zn concentration in chickpeas grown in North America range from 2.5 mg/100 g (Ray et al. 2014) to 5.3 mg/100 g (Thavarajah and Thavarajah 2012a, b). Nongenetic sources of variance, including environment, year, and their interactions, have shown greater effect magnitudes than the genetic variance for several important minerals of global concern, including Fe, Mg, and Zn (Ray et al. 2014; Vandemark et al. 2018).

Chickpea is a rich source of prebiotic carbohydrates, SAs, FOSs, and RFOs (Peterbauer and Richter 2001; Vandemark et al. 2019). Seed concentrations of selected prebiotic carbohydrates were determined for adapted kabuli breeding lines and cultivars grown in the United States in Washington and Idaho (Vandemark et al. 2019). Sucrose was the most abundant low digestible carbohydrate in chickpea seed (≈1700 mg/100 g), followed by the FOS stachyose (≈1200 mg/100 g) and the SA sorbitol (≈700 mg/100 g). The least abundant low digestible carbohydrates in chickpea seed were fructose (≈2 mg/100 g) and mannitol (≈13 mg/100 g). Genotype effects were significant for fructose, sucrose, raffinose, and kestose, and environmental effects were significant for several prebiotic carbohydrates. However, the year effect was the greatest source of variance for all carbohydrates. The concentrations of most carbohydrates were significantly lower in 2018 than in

Table 9.2 Seed protein concentrations (%) for chickpea breeding lines and cultivars grown at Pullman, WA, and Genesee, ID in 2018

Entry	Pullman[a]	Genesee	Combined
CA13900046C	24.0 a	23.8 a	23.9 a
CA13900149C	23.6 ab	22.7 abc	23.2 ab
CA13900049C	21.5 cde	24.9 ab	22.4 ab
CA0790B0043C	22.7 abcd	21.3 abc	22.0 ab
CA13900119C	22.2 abcd	21.0 abc	21.7 ab
CA13900147C	22.4 abc	20.9 abc	21.6 ab
Sierra	23.1 abc	20.0 abcd	21.6 ab
CA13900151C	22.2 bcd	20.7 abc	21.4 ab
CA0890B0429C	22.2 abcd	20.3 abc	21.3 ab
CDC Frontier	22.5 abcd	19.6 abcd	21.0 abc
Billy Beans	21.6 cde	20.5 abc	21.0 abc
CA13900023C	22.2 bcd	19.9 abcd	21.0 abc
CA13900162C	21.8 cd	20.2 abcd	21.0 abc
Royal	22.4 abcd	19.2 bcd	20.8 abc
CA0790B0547C	21.9 bcd	19.4 bcd	20.7 abc
CA13900129C	21.8 cd	19.2 bcd	20.5 bc
CA13900002C	21.4 cde	10.0 bcd	20.2 bc
CA13900139C	21.2 de	17.9 cd	19.9 bc
CDC Orion	20.0 e	15.9 d	17.9 c
Grand Mean	22.2	20.2	21.2

[a]Means within a column followed by the same letter are not significantly different (Tukey's HSD $\alpha = 0.05$)

2017, the growing season of the latter being characterized by less precipitation and greater heat stress during grain filling. These results likely reflect the role many prebiotic carbohydrates, including RFOs (Panikulangara et al. 2004) and SAs (Abebe et al. 2003), have as osmoprotectants produced in response to high temperature and drought stress.

Increasing protein content in chickpea seeds has been proposed to be the most important research area in chickpea breeding and genetics (Upadhyaya et al. 2016). Estimates of protein concentration in chickpea seed vary from approximately 12–29%, depending on the materials evaluated. However, recent estimates of seed protein concentration in diverse chickpea materials have averaged about 18% (Upadhyaya et al. 2016) and 20% (Jadhav et al. 2015). In 2018, Dr. Vandermark's breeding program evaluated seed protein concentration in 19 advanced chickpea breeding lines and cultivars grown at 2 locations. Significant differences were detected between entries for protein concentration, which ranged from approximately 18 to 24% (Table 9.2). Unfortunately, a relatively large negative correlation ($r \approx -0.7$) was observed between seed yield and seed protein concentration, which is troubling given the importance of both traits. Recent studies with adapted kabuli chickpeas suggest genetic effects on chickpea nutritional qualities are minor compared to the nongenetic impacts (Ray et al. 2014; Vandemark et al. 2018, 2019). Seed yield and protein concentration are negatively correlated in adapted kabuli chickpeas (Table 9.2). Evaluation of more genetically diverse materials appears

necessary for developing improved cultivars with appreciably enhanced nutritional qualities. An excellent diversity panel for detecting variation in chickpea is a mini-core collection (Upadhyaya and Ortiz 2001) representing approximately 10% of the ICRISAT chickpea core collection (1956 accessions).

6 Conclusions

Every human requires ~50 essential nutrients for healthy living. Cereal-based diets are a good source of carbohydrates as well as the energy that can satisfy daily caloric requirements. However, cereals do not provide the recommended dietary intake of protein and micronutrients such as Fe, Zn, vitamins A and C, riboflavin, Se, copper (Cu), calcium (Ca), folates, and carotenoids. Proteins and micronutrients are not only essential for general well-being but are also necessary for disease prevention. Pulse crops (lentil, field pea, and chickpea) are a central part of the diet for many communities worldwide, and it provides a significant amount of nutrients. Therefore, pulse crops provide a better whole-food solution to combat all forms of malnutrition.

Biofortification of crops is more nuanced than just increasing grain concentration for a given nutrient. All genome-wide association studies noted herein utilized small experimental populations that may not completely represent pulse crop diversity. Additionally, some studies only use data from one growing season, which is insufficient to dissect genotype × environment interactions for these traits (Thavarajah et al. 2014, 2015a, b). Biofortification efforts must include selection for increased nutrient bioavailability with moderate levels of antinutritional factors. The development of cultivars that maintain adequate nutrition levels after processing (freezing and cooking) is also necessary. For example, processing field pea can result in a 30% decrease in grain Zn composition and a 17% increase in phytic acid-bound Zn (Poblaciones and Rengel 2017). Other challenges related to the success of a biofortified crop include the following: (1) the crop must be high yielding and profitable; (2) the crop must alleviate malnutrition symptoms; and (3) the farmer and consumer must be willing to adopt the biofortified crop (Bouis and Welch 2010).

Biofortification of pulses is possible. Several varieties of biofortified pulse cultivars have been released by HarvestPlus and ICARDA, as well as in cooperation with local governments such as the Rwanda Agriculture Board (Bouis and Saltzman 2017). Nutritional breeding of pulses shows promise and will advance as scientists continue to emphasize the importance of these pulse crops for human nutrition and sustainable agriculture. Moreover, pulse crops are excellent nitrogen (N) fixers, providing 75–120 kg of N per hectare. Enhancing the nutritional value of pulse crops will undoubtedly improve human nutrition worldwide, as well as provide N and carbon benefits to subsequent cereal crops.

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Dry Bean Biofortification with Iron and Zinc

10

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Abstract

Dry beans, a nutrient-dense dietary staple in Africa, Latin America, and the Caribbean, deliver nutrients such as protein, minerals, and folate, which are often in short supply in other staples. Beans are relatively rich in iron and zinc, two micronutrients for which dietary deficiencies impact billions of people globally. Wide genetic variability in beans seeds, from ~34 to 96 mg/kg for iron and 21 to 59 mg/kg for zinc, led to the recognition that biofortification of beans for maximum levels of these micronutrients is possible through plant breeding. Biofortification efforts to develop bean varieties with seed iron concentrations approaching 90 mg/kg have been underway since the early 2000s. Iron and zinc levels in seeds are positively correlated with each other, and although iron has been the major focus of biofortification efforts, zinc is often evaluated alongside iron. Germplasm diversity screenings have revealed multiple high iron sources in cultivated Andean and Middle American beans as well as wild *P. vulgaris* and genotypes from closely related species *P. dumosus*, *P. acutifolius*, and *P. parvifolius*. Both seed iron and zinc are moderately heritable traits, and breeding with high iron donor parents based on phenotypic selection has been successfully utilized to achieve genetic gains. To date, at least 60 high iron bean varieties have been released over 12 countries in Eastern and Southern Africa and Latin America. Bean breeders have combined the high iron trait with

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other traits important to farmers, including seed yield, disease resistance, and abiotic stress tolerance. The application of genomic approaches in breeding high iron beans has been limited. While numerous seed iron and zinc Quantitative Trait Loci (QTL) studies have been undertaken and a meta-analysis identified 12 meta-QTL, 8 of which are for both increased iron and zinc, there has not been much traction in incorporation of these QTL in breeding strategies. Since iron and zinc are quantitative traits controlled by many small-effect QTL, breeders have not found marker-assisted breeding with single or multiple QTL worthwhile. A genomic prediction approach, which in contrast, utilizes thousands of random markers throughout the genome, may be a promising strategy to apply to breeding high iron and zinc beans, and is currently being explored. The prospect of using a transgenic approach to develop high iron and zinc beans is limited at this time due to challenges with plant regeneration and public acceptance of genetically modified (GMO) beans, which may change in the future, and there are many potential candidate genes. The future of biofortification of beans with iron must also look beyond a pure focus on increasing concentration as this approach relies on the assumption that higher iron yields deliver more absorbable iron. To date, one human efficacy study has demonstrated a positive, although slight, effect of biofortification on human iron status. Regardless of concentration, iron from beans can have very low bioavailability due to seed coat polyphenols and phytic acid present in the cotyledons. Evidence from in vitro and animal studies suggests that beans without inhibitory polyphenols and with promoter polyphenols would have higher iron bioavailability and thus deliver more iron. Therefore, redefining biofortification to focus on both iron bioavailability and iron concentration simultaneously in breeding programs has the potential to deliver substantially more nutritional benefits to consumers. The introduction of varieties labeled as high iron beans in Africa and Latin America has largely been met with interest and adoption by farmers and consumers due to strong promotion and the development of varieties with superior yield and disease resistance. Going forward in addition to focusing on iron bioavailability, a greater focus should also be placed on zinc.

Keywords

Fe fortification · Zn fortification · Dry beans · QTL · Biofortified varieties

1 Introduction

1.1 Economic Importance of Dry Bean

Dry bean (*Phaseolus vulgaris* L.) is the most widely produced grain legume worldwide, following soybean and groundnut, and is the first grain legume for direct human consumption. Major dry bean-producing countries include Brazil, the USA, Uganda, Mexico, and Tanzania (FAOSTAT 2020). According to 2017 FAO food

availability data, the highest bean consumption occurs in East Africa, followed by Central America, and Brazil (FAOSTAT 2020). Top bean-consuming countries of Rwanda, Uganda, and Tanzania derive approximately 322–161 kcal and 20.8–10.5 g of protein daily from beans (FAOSTAT 2020). The top bean-consuming countries of Latin America and the Caribbean, including El Salvador, Brazil, Cuba, Nicaragua, Honduras, and Guatemala, derive approximately 157–115 kcal and 10.2–7.5 g of protein daily from beans (FAOSTAT 2020).

Dry beans are comprised of 60–70% carbohydrates of which 40–52% is starch, 24–27% is fiber, 20–29% protein, 2–4.5% ash, and 1–% fat (Katuuramu et al. 2018; Sathe et al. 1984; Chen et al. 2016; Pujolà et al. 2007). The minerals contained in the ash fraction include macro- and micronutrients, and in descending order include macronutrients: potassium, phosphorus, sulfur, magnesium, and calcium; and micronutrients: iron, zinc, manganese, boron, and copper (McClean et al. 2017). Dry beans also are a rich source of folate, this is in contrast to grains including rice, corn, and wheat, which are low in folate (Jha et al. 2015; Strobbe and Van Der Straeten 2017).

Dry beans' nutrient profile is a value to consumers, especially to people with limited dietary diversity who rely on starchy staples to meet most of their calorie needs. Beans are rich in iron and zinc, two micronutrients with major global human dietary deficiencies. Iron deficiency affects 2 billion people globally including >50% women and preschool children in developing countries and causes 20% of death in women during child birth (Zimmermann and Hurrell 2007). An estimated 17.3% of humans worldwide are at risk for insufficient zinc intake (Wessells and Brown 2012). Zinc-deficiency symptoms include stunted growth in children and infants and impaired vitamin A use and vitamin D function (Maxfield and Crane 2019). Recommended dietary allowance (RDA) are intake levels that meet the requirements of almost all healthy individuals in an age and gender group, and these range from 0.27 to 27 mg/day/iron and 2 to 13 mg/day/zinc (Devaney and Barr 2002; Trumbo et al. 2001) (Table 10.1). A single 100 g serving of cooked beans contributes approximately 2.3 mg of iron and 1.06 mg of zinc (Marinangeli et al. 2017).

Thus, iron and zinc are critical micronutrient deficiencies with potential to be addressed through biofortification. Dry bean was chosen for iron and zinc biofortification mainly because of the high levels of iron-deficiency anemia in regions where beans are produced and consumed and the natural genetic variability for concentrations of these micronutrients within the species (Asare-Marfo et al. 2013). Beans were also chosen because they have higher concentrations of iron and zinc than in cereals. For example, compared to milled rice, common beans have about 4–10 times the amount of iron and 2–3 times the amount of zinc (Blair et al. 2009; Bouis 2018).

HarvestPlus is the entity overseeing and driving the biofortification efforts of bean and all crops. It is part of the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH) and works collaboratively with institutions globally in development, testing, and release of biofortified crops, with CGIAR International Center for Tropical Agriculture (CIAT) at headquarters in Colombia and a station in

Table 10.1 Recommended dietary allowances (RDAs) for iron and zinc in milligrams[a]

	Iron (mg)				Zinc (mg)			
Age	Male	Female	Pregnancy	Lactation	Male	Female	Pregnancy	Lactation
Birth–6 months	0.27[b]	0.27[b]			2[b]	2[b]		
7–12 months	11	11			3	3		
1–3 years	7	7			3	3		
4–8 years	10	10			5	5		
9–13 years	8	8			8	8		
14–18 years	11	15	27	10	11	9	12	13
19–50 years	8	18	27	9	11	8	11	12
51+ years	8	8			11	8		

Adapted from https://ods.od.nih.gov/factsheets/Iron-HealthProfessional/#h6 and https://ods.od.nih.gov/factsheets/Zinc-HealthProfessional/

[a]RDAs for vegetarians are 1.8 times higher

[b]Represents adequate intake (AI) that is equivalent to the mean intake of iron in healthy, breastfed infants

Uganda as leaders in the breeding efforts (Andersson et al. 2017). The original target at the start of the Harvest Plus Initiative was for someone to receive about 30% of the daily estimated average requirement (EAR) of iron and 40% of the EAR for zinc from a 100 g daily serving of beans (Petry et al. 2015; Bouis and Welch 2010). EAR is the median intake needed to meet the requirements of half the healthy individuals in an age and gender group. From the outset, increasing the concentration of iron and zinc in bean seeds through plant breeding was seen as the way to achieve biofortification. A note on the presentation of dietary requirement values as compared to amounts in seeds; they are typically expressed differently in the literature. While dietary contributions are described as a 100 g serving of cooked beans (which are approximately 50% water), plant science literature describes iron and zinc concentrations on a dry weight basis, usually in mg in a kg of dry beans, uncooked, without water. Therefore, there are conversions and assumptions needed when going between these two measures, taking into account water uptake during cooking, nutrients lost during cooking, and seed size; these assumptions will be discussed in greater detail at the end of Sect. 4.

Plant breeding targets for iron and zinc have been set based on concentrations in raw beans (uncooked concentrations). For iron, 94 mg/kg was set as the target to be defined as "biofortified." This was determined as an 80% increase over a baseline iron concentration in beans of 50 mg/kg based on early germplasm screening (Bouis and Welch 2010; Islam et al. 2002). For zinc, the initial goal was to increase 17 mg/kg above the observed local levels (Bouis and Welch 2010). While iron and zinc are commonly evaluated simultaneously in the breeding programs, iron has been the frontrunner in terms of development, promotion, and human efficacy studies with biofortified beans. Zinc biofortification of beans has taken a backseat, and while not completely forgotten, it is just handled more as a bonus after high iron levels are achieved.

1.2 Evaluation of Genetic Resources for Grain Micronutrient Concentration

The genus *Phaseolus* consists of approximately 70–80 species, all native to the Americas (Gepts 2014). Five of these species have been domesticated, including, listed in order of economic importance, *P. vulgaris* (common bean), *P. lunatus* (lima bean), *P. coccineus* (scarlet runner bean), *P. acutifolius* (tepary bean), and *P. dumosus* (year bean). *P. vulgaris* originated ~4 million years ago in Middle America, most likely in central Mexico (Bitocchi et al. 2012; Delgado-Salinas et al. 1999), and was domesticated independently in two locations, Middle America and the Andes, with a bottleneck in the Andes before domestication (Bitocchi et al. 2012). The primary gene pool of *P. vulgaris* consists of domesticated and wild beans from both the Middle American and Andean gene pools. The wild germplasm within the species ranges from Chihuahua in northern Mexico to Cordoba in Argentina, a distance of 10,000 km (Gepts et al. 2020; Bitocchi et al. 2017). In spite of the large

range, the species is adapted to middle altitudes, moderate temperatures and rainfall, predominately self-pollinated, and with annual life cycles (Gepts et al. 2020).

The secondary gene pool includes *P. coccineus* and *P. dumosus*, both species distributed in Middle America and adapted to cool and humid climates, predominately cross-pollinated with perennial life cycles. *P. coccineus* is adapted to higher altitudes than *P. dumosus* (Bitocchi et al. 2017). The secondary gene pool also includes the wild species *P. costaricenses* (Gepts et al. 2020). The tertiary gene pool includes *P. acutifolius*, which is an annual adapted to hot and dry climates, and wild forms are found in the southwestern US to central Mexico (Blair et al. 2002).

There have been multiple large-scale efforts to evaluate the primary, secondary, and tertiary gene pools for seed Fe and Zn concentration. The first evaluation was made of the CIAT core collections of 1072 cultivated accessions and 119 wild accessions (Islam et al. 2002; Beebe et al. 2002). The ranges of iron in the cultivated lines were from 34 to 92 mg/kg and up to 96 mg/kg in the wild beans. The ranges of zinc were from 21 to 59 mg/kg in the cultivated and up to 43 in the wild beans (Islam et al. 2002; Beebe et al. 2002). In this screening, it was found that beans from the Middle American gene pool had slightly lower (~2 mg/kg less) average levels of Fe as compared to Andean and admixed lines and that Andean beans had slightly lower levels (~2 mg/kg less) of Zn as compared to Middle American and admixed lines (Islam et al. 2002).

Since the first germplasm screening at CIAT, an additional 12 published large-scale studies have evaluated the diversity of seed Fe and Zn levels in bean germplasm collections from 50 up to 1512 genotypes in a study. These studies come from 10 countries including Brazil, the USA, Rwanda, Uganda, Tanzania, DR Congo, Portugal, Italy, Croatia, and India, Most of the studies evaluate local germplasm including landraces and improved varieties, either collected from farmers or from germplasm banks. Three studies also evaluated the germplasm in more than one location or across multiple seasons (McClean et al. 2017; Delfini et al. 2020; Philipo et al. 2020a). A list of the studies is found in Table 10.2. Almost every study was able to identify beans with iron levels at least as high as what was found in the CIAT germplasm screening, and four studies, two from Brazil, one from Tanzania, and one from DR Congo, found germplasm with Fe levels higher up to 150 plus mg/kg (Delfini et al. 2020; Mbikayi et al. 2018; Ribeiro et al. 2013; Philipo et al. 2020a, b). Only one study, however, from Tanzania, found germplasm with Zn levels higher than what was found in the initial CIAT core collection screening (Philipo et al. 2020b). Based on these many germplasm screenings, it appears that there are numerous potential sources of high Fe donor parents, but not as many or at the same range for Zn, and this likely deterred breeders from working to increase Zn levels. Both the genotype and the growing environment are factors important in determining the seed Fe and Zn concentrations; therefore, the higher levels observed in some studies are likely due to a combination of these factors.

Table 10.2 Genetic diversity studies for seed iron and zinc concentration

Country	Location	#[a]	Genotype source	Method[b]	Iron			Zinc			Study reference
					Mean	Low	High	Mean	Low	High	
					(mg/kg) in seeds						
Africa											
DR Congo	Kinshasa	383	Farmers' fields in Eastern Congo	ICP	72	40	124	31	10	65	Mbikayi et al. (2018)
Rwanda	gene bank	365	Great Lakes region of Central Africa and Rwanda "Seeds of Hope"	AAS	68	45	98	35	25	49	Blair et al. (2010d)
Tanzania	Morogoro (screenhouse)	90	Tanzania; collected from farmers and markets	AAS	55	24	78	31	19	56	Tryphone and Nchimbi-Msolla (2010)
	Arusha	99	Landraces and cultivars	AAS	59	20	151	24	16	33	Philipo et al. (2020a)
	Morogoro	99		AAS	52	14	142	32	15	50	
	Mbeya	99		AAS	58	18	138	38	15	65	
Uganda	Kawanda	61	Rwanda Seeds of Hope Landraces	XRF	71	56	89	33	24	38	Amongi et al. (2018)
		61		XRF	75	63	93	40	32	47	
Asia											
India	Jammu, Kashmir	51	Germplasm collection	ICP-OES	18	7	72	8	4	19	Mahajan et al. (2015)
Europe											
Croatia	Zagreb	226	Croatian landraces	AAS	72	64	76	27	25	29	Palčić et al. (2018)
Italy	Perugia	178	Mostly European landraces	EDXRF	62	38	94	31	19	44	Caproni et al. (2020)
Portugal	Oeiras	155	Portuguese landraces	AAS	–	32	88	–	12	45	Pinheiro et al. (2010)

(continued)

Table 10.2 (continued)

Country	Location	#[a]	Genotype source	Method[b]	Iron			Zinc			Study reference
					Mean	Low	High	Mean	Low	High	
					(mg/kg) in seeds						
North America											
United States	Colorado	277	Middle American from N and Central Am. Race Durango and Mesoamerican	ICP-OES	58	43	81	27	17	35	McClean et al. (2017)
	Michigan	277		ICP-OES	58	36	90	28	19	39	
	Nebraska	277		ICP-OES	59	44	79	27	19	39	
	North Dakota	277		ICP-OES	–	–	–	26	18	38	
	Michigan	206	Andean Diversity Panel	ICP-OES	74	55	99	32	22	40	Katuuramu et al. (2018)
South America											
Brazil	Santa Maria	50	Cultivars	ICP-OES	90	57	159	29	22	35	Ribeiro et al. (2013)
	Pato Branco, Lapa, PontaGrossa	1512	IDR-IAPAR-EMATER germplasm bank, local, commercial	ICP-OES	80	52	115	36	26	47	Delfini et al. (2020)
Colombia	Dept. of Valle	1031	CIAT cultivated core collection	ICP-OES	55	34	89	35	21	54	Beebe et al. (2002)
		119	CIAT wild core collection	ICP-OES	60	–	96	29	–	43	
Colombia	Dept. of Valle	1072	CIAT core collection	ICP-OES	55	35	92	34	21	59	Islam et al. (2002)

[a]Number of genotypes evaluated

[b]Method of analysis to determine seed Fe and Zn concentration, all studies reported raw seed minerals, except Katuuramu et al. (2018), where cooked seed minerals were reported

2 Genotype by Environment Interactions (G×E)

Seed iron levels are influenced by genotype and environment; there is also a significant G×E that is often observed by lack of correlation of iron levels from one location to the next in multilocation trials (McClean et al. 2017). One of the major abiotic stresses of importance to bean production is drought, and its incidence is expected to become more prevalent in many major bean-growing regions with climate change (Ramirez-Cabral et al. 2016). The effects of drought on seed Fe and Zn have been variable. In a study with 20 African bean genotypes grown under drought conditions chosen to mimic those expected in southeastern Africa by 2050 as a result of climate change, iron levels modestly decreased from an average of 59 mg/kg in the well-watered to 54 mg/kg in the drought conditions. A few varieties had increased seed Fe in the drought treatment. Zinc levels slightly increased under drought, and phytic acid went from 0.91% to 1.16% of seed weight (Hummel et al. 2018). A study of 81 genotypes of the Middle American Diversity Panel grown in three US locations under drought versus nondrought side by side found that Fe was 71 mg/kg in drought and 65 mg/kg in nondrought, this difference was significant at a *p*-value of 0.02. The drought treatment reduced yields by almost half, and seeds were about 10% smaller (McClean et al. 2017). In a study of 21 Middle American genotypes, drought stress overall was not significantly related to seed Fe or Zn concentration, but when looking at individual varieties, six of the genotypes had decreased Fe concentration under drought, four did not change, and two increased (Smith et al. 2019). Seed phosphorus levels increased under drought, and since phytic acid is the major storage form of phosphorus in bean seeds and an inhibitor of Fe bioavailability, the nutritional relationship on how drought influences Fe bioavailability should be considered further (Smith et al. 2019).

3 Brief on Diversity Analysis

Through germplasm screening a number of inter-gene pool landraces from the northern Andes (G14519, G21242, G23823, and G23834) were found to have high Fe and have been useful in breeding for both Andean and Middle American bean market classes (Beebe 2012). Additionally, some wild beans have been identified with high iron; G10022, from Mexico, has 108 mg/kg Fe (Blair and Izquierdo 2012). Challenges in working with wild beans include small seed size, climbing growth habit, and long growing cycle. An advanced backcross scheme was used by Blair and Izquierdo (2012) to attempt to transfer and map the high Fe to an Andean large red bean background via advanced backcross line development. In total, 138 BC2F2:5 lines were developed within the population, and Fe concentrations were skewed to the low end, with most even lower (negative transgressive segregation) than the recurrent parent, and none approaching the level of the wild bean. In the case of zinc, there was also negative transgressive segregation. The highest Fe progeny had 8–19% more Fe than the recurrent parent depending on planting location (Blair and Izquierdo 2012). Another wild × cultivated cross

between G22387 and Bayo Baranda used 120 F2:3 lines. Significant positive transgressive segregation for Fe (41–142 mg/kg, where the parents were 45 and 71 mg/kg) and Zn (27–67 mg/kg, where the parents were 20 and 36 mg/kg) was found in this case, and neither Fe nor Zn concentrations were correlated with seed size (Guzmán-Maldonado et al. 2003).

The secondary and tertiary gene pools have been used as parental sources to improve seed Fe and Zn concentration. In this regard, related species *P. dumosus* and *P. acutifolius* have been used in mating designs with 2–3 high iron parents (Mulambu et al. 2017). A *P. dumosus* accession with 127 mg/kg Fe G35575 was identified in a germplasm screening (Beebe 2012). Interspecific crosses with *P. coccineus* have also been used to increase Fe concentration (Blair 2013). One high iron bean from Guatemala ICTA Superchiva derives its high iron from *P. parvifolius*, a wild species closely related to tepary bean (Beebe 2020; Gujaria-Verma et al. 2016). Beebe (2020), in a recent perspective on Fe biofortification in beans, suggests that the adaptation of *P. parvifolius*, *P. dumosus,* and *P. acutifolius* to arid climates and high pH soils may lead to mechanisms that allow them to better extract iron from these soils (Beebe 2020).

4 Classical Genetics and Traditional Breeding

4.1 Phenotyping Methodology

The primary breeding goal of bean biofortification has been to increase total seed Fe concentration. In order for a bean to be Fe biofortified according to HarvestPlus definition, it has at least 22 mg/kg more than the locally consumed varieties (Andersson et al. 2017). This objective has been pursued primarily through phenotypic evaluation of total raw seed iron levels, typically in F_3 generations or later. There are three common methods employed for Fe and Zn: (1) inductively coupled plasma—optical emission spectrometry (ICP-OES, (2) atomic absorption spectroscopy (AAS), and (3) X-ray florescence (XRF). ICP-OES and AAS methods require moderate sample preparation, including cleaning, milling, and digesting of samples with a strong acid. The analysis instruments are specialized, expensive, and require significant investment in time and personnel (Guild et al. 2017a). One positive aspect of this method is that there are many service labs set up with this equipment and to perform this analysis. Another positive, and this goes for all the methods, is that since iron and zinc are elements, there is no need to be concerned about storage and handling, or postharvest changes in composition. The major area of concern however is contamination. Fe contamination may occur from soil or dirt on the samples, and Zn contamination may occur from some of the milling and processing plastics that may contain zinc (Guild et al. 2017b). Care should be taken to clean samples prior to analysis, and contamination can be checked for by measuring aluminum levels in samples, where anything greater than 5 mg/kg is a sign of contamination (Guild et al. 2017b). XRF is more useful as a high-throughput method; no digestion is needed, but bean samples still need to be milled into flour. Validation results show

that XRF is expected to be within ±5.5 mg/kg for Fe and ±3.5 mg/kg for Zn values from ICP-OES (Guild et al. 2017a).

4.2 Inheritance Studies

Mukamuhirwa et al. (2015) studied the inheritance of seed Fe and Zn based on these standard phenotyping methods in a full diallel mating design with six genotypes, three high, and three low Fe and Zn. The seed concentrations of both Fe and Zn were controlled by both additive and nonadditive effects. Narrow-sense heritabilities (the genetic variance that is additive) were moderately high for Fe at 0.71 and Zn at 0.83. In other studies, broad-sense heritabilities of seed Fe were 0.68 and seed Zn were 0.84 (McClean et al. 2017), and narrow-sense heritabilities were 0.67 and seed Zn was 0.47 (Diaz et al. 2020). The moderate heritability values reported for Fe and Zn across these three studies suggest that phenotypic selection in segregating generations will be moderately effective to improve Fe and Zn concentrations (Mukamuhirwa et al. 2015). However, two of the high Fe and Zn lines used as parents had a negative general combining ability and actually contributed to a reduction in Fe and Zn concentration in the progeny, and this is an indication of some level of epistasis and/or overdominance and is an indication of caution that may be needed in choosing parental lines and employing particular breeding strategies. One other important finding from the work of Mukamuhirwa et al. (2015) was that reciprocal crosses gave different outcomes for both Zn and Fe, and the best strategy would be to use the high Fe and Zn lines as maternal parents (Mukamuhirwa et al. 2015).

Transgressive segregation for seed Fe and Zn has been observed, more so for wide crosses versus narrow crosses closely related genotypes within the same market class or gene pool (Blair 2013).

4.3 Classical Breeding Achievements

The early stages of the breeding process, including parental selection, mating, and early generation selection, have largely been carried out by CIAT, at breeding stations in Colombia and Uganda. Later stages of the breeding process, including advanced line selection for local adaptation, agronomics, and quality, have been conducted by national program partner institutions in eight countries in East and Southern Africa and nine countries in Central and South America (Andersson et al. 2017; Saltzman et al. 2017). Dry bean iron biofortification breeding activities were begun at CIAT in 2003 (Mulambu et al. 2017). In addition to the HarvestPlus-directed bean biofortification programs, the Pan African Bean Research Alliance (PABRA) has released high Fe beans in Burundi, Malawi, Kenya, and Zimbabwe (Andersson et al. 2017). In East Africa, a regional breeding approach was begun in 2001. First, a set of 70 already popular landraces and varieties from DRC, Ethiopia, Kenya, Sudan, and Uganda were evaluated for Fe and Zn. Some superior lines were

identified and distributed to 25 countries in Africa for breeding use, agronomic evaluation, and some were released (Kimani 2005).

Breeding strategies using multiparent populations have been applied to biofortification at the University of Nairobi since 2005 (Kimani and Warsame 2019). The goal of the breeding strategy was to incorporate high Fe and Zn along with many important traits, including abiotic and biotic stress resistance important in the production region, such as angular leaf spot, root rot, anthracnose, and low soil fertility. Two parent crosses were made and then F_1 hybrids were crossed to each other, then the resulting F_1 was planted for evaluation or crossed to another well-adapted cultivar to produce a three-way cross (Singh 1994). The final cross in the series was always back to the high mineral parent as the female. The multiparent breeding strategy has resulted in multiple lines with Fe levels above the iron levels of the parental lines (i.e., up to 136 mg/kg) in combination with good agronomic qualities (Kimani and Warsame 2019).

The first official biofortified bean germplasm release was in 2010 of two high iron and zinc red mottled beans (NUA35 and NUA56) developed by CIAT. These lines were developed through early generation selection for seed Fe and Zn in a backcross population of ['CAL96' × (CAL96 × G14519)]. Cal96 is a common red mottled variety in East Africa, and G14519 is the donor of the high Fe and Zn. The NUA lines had on average 18–23 mg/kg more Fe and 8 and 7 more mg/kg of Zn than CAL96 across the 15 locations where they were grown (Blair et al. 2010a).

HarvestPlus high iron beans have been released in at least 12 countries, including 8 countries in Latin America and the Caribbean and 4 countries in Africa, with approximately 60 biofortified varieties released, including at least 26 in Africa and 18 in Latin America and the Caribbean (Table 10.3) (Saltzman et al. 2017). The biofortified varieties typically include other traits of interest that benefit farmers, such as increase seed yield and disease resistance (Andersson et al. 2017). Recent efforts in biofortified beans have focused on the need to add drought tolerance and multiple disease resistances (Kimani and Warsame 2019).

4.4 Breeding Challenges

A few of the challenges of breeding for increased iron concentration include that the trait is invisible and has a moderate heritability. The trait is influenced by environmental production conditions, especially soil conditions, which may result in some biofortified varieties not exhibiting their high iron trait in some growing environments. The trait is also prone to genotype by environment interactions such that depending on the growing environment, a nonbiofortified variety may in some cases have higher iron than a biofortified variety. Many traits have moderate heritability and a prevalence of genotype × environment interactions, seed yield for example. These challenges are being addressed in part by setting baseline iron levels for target increases according to local preferred varieties grown in the same region instead of assuming a global baseline. Mbikayi et al. (2018) studied 383 genotypes collected from farmers' fields in Eastern Congo from 2006 to 2012,

Table 10.3 Iron biofortified bean variety releases

Country	Variety	Release year	Origin	Seed type	Plant growth habit	Seed Fe (mg/kg)	Potential high Fe parental source[a]
Bolivia	Fortaleza	Before 2016	CIAT	n/a	Bush	90	G14519
Brazil	BRS Agreste	2009	EMBRAPA	Brown	Semi-climber	90	
	BRS 9435 Cometa	2007	CIAT	Carioca	Climber	90	
	BRS Pontal	2004	EMBRAPA	Carioca	Bush	90	
Colombia	Corpoica Rojo 39	2015	CIAT	Red	Bush	86	G10022, G23818B, G23823E, G40102, FEB 226
	Corpoica Rojo 43	2015	CIAT	Red	Bush	95	G10022, G23818B, G23823E, G40102, FEB 226
	BIO-101	2016	CIAT	Red	Bush	83	G10022, G23818B, G23823E, G35575A
	BIO-107	2016	CIAT	Red	Bush	82	
	BIO-102	2019	CIAT	Red mottled	Climber	86	G40102, FEB 226
DR Congo	COD MLV 059	2012	INERA	Red mottled	Climber	84	
	PVA 1438	2013	CIAT	Red kidney	Bush	79	
	Nain de Kyondo	2013	DR Congo	White	Climber	76	
	COD MLB 032	2013	INERA	Sugar	Bush	76	
	Cuarentino	2013	DR Congo	White	Climber	100	
	COD MLB 001	2012	INERA	Red mottled	Bush	66	
	HM 21-7	2012	CIAT	Red mottled	Bush	62	

(continued)

Table 10.3 (continued)

Country	Variety	Release year	Origin	Seed type	Plant growth habit	Seed Fe (mg/kg)	Potential high Fe parental source[a]
	RWR 2245	2011	Rwanda	Red mottled	Bush	66	
	VCB 81013	2008	CIAT	White	Climber	69	
	NUA 100	After 2016	CIAT	n/a	n/a	n/a	G14519
	MNC 488-2	after 2016	CIAT	n/a	n/a	n/a	G10022, G21242
	NUV 119-4	after 2016	CIAT	n/a	n/a	n/a	G14519
	NUA 99	after 2016	CIAT	Red mottled	n/a	n/a	G14519
	Namulenga	2013	CIAT	Zebra	Climber	76	
El Salvador	CENTA Ferromás	2011	CIAT	n/a	Bush	80	G23818B, G40102
Guatemala	ICTA Superchiva	2014	ICTA	Black	Bush	74	G40102
	ICTA Peten	2010	ICTA	Black	Bush	76	
	ICTA Chorti	2016	CIAT	Black	Bush	90	G10022, G23823E, G35575B, FEB 226
Honduras	MIB (NUT) 396-33	2016	CIAT	n/a	Bush	78	
	MIB (NUT) 397-72	2016	CIAT	n/a	Bush	71	
Nicaragua	INTA Ferroso	2014	CIAT	n/a	Bush	84	G23818B, G40102,
	INTA Nutritivo	2012	CIAT	n/a	Bush	68	G23818B, G40102
	INTA BIOF100	2017	CIAT	n/a	n/a	n/a	G10022, G23818B, G23823E, G40102, FEB 226, G35575A

	INTA Rojo Bio-Apante	2019	CIAT	red	n/a	n/a	G10022, G23823E, G35575B, FEB 226,
	INTA BIODOR	2017	CIAT	n/a	n/a	n/a	G10022, G23823E, G 35575A, FEB 226
Panama	IDIAP NUA 24	2012	CIAT	n/a	Bush	78	G14519
	IDIAP NUA 27	2012	CIAT	n/a	Bush	n/a	G14519
Rwanda	MAC 44	2010	CIAT	Red mottled	Climber	78	
	RWV 1129	2010	RAB	Salmon	Climber	77	
	CAB 2	2010	CIAT	White	Climber	81	
	RWR 2245	2010	RAB	Red mottled	Bush	76	
	RWR 2154	2010	RAB	Sugar	Bush	71	
	RWV 3316	2012	RAB	Red	Climber	87	
	RWV 3006	2012	RAB	White	Climber	78	
	RWV 3317	2012	RAB	Sugar	Climber	74	
	MAC 42	2012	CIAT	Sugar	Climber	91	
	RWV 2887	2012	RAB	Dark red	Climber	85	
Uganda	MAC 44	2016	CIAT	Red mottled	Climber	78	
	RWR 2245	2016	RAB	Red mottled	Bush	76	
	RWR 2154	2016	RAB	Sugar	Bush	71	
	MORE 88002	2016	CIAT	Yellow	Bush	70	
	Nyiramuhundo	2016	Rwanda	Yellow/ orange	Climber	67	
	Roba 1	2013	CIAT	Beige	Bush	64	

(continued)

Table 10.3 (continued)

Country	Variety	Release year	Origin	Seed type	Plant growth habit	Seed Fe (mg/kg)	Potential high Fe parental source[a]
Zimbabwe	Jasmine	after 2016	CIAT	Navy	n/a	n/a	G10022, G23823E, FEB 226

Data gathered primarily from Andersson et al. (2017) and Beebe (2020) and various variety release reports and references to these varieties in the HarvestPlus reports and peer-reviewed literature. This is not a complete list as recent reports from HarvestPlus indicate 60 high iron bean releases

[a]From Beebe (2020)

at a time when biofortified beans were just beginning to be adopted by farmers. They grew these seeds in a single-field site in Congo and measured seed Fe and Zn. They then determined what percentages were meeting the biofortification standard of 44 mg/kg above baseline and found that 21% were at the baseline level of 50–60 mg/kg, 31% were at 60–70 mg/kg, 21% at 70–80 mg/kg, 11.8% at 80–90, 10.7% at greater than 94 mg/kg, and 3.4% below baseline.

Another challenge is ensuring an actual nutritional value is passed on to consumers eating high iron beans. So far, evidence suggests that biofortification is working as intended with beans, although the results with biofortified rice and pearl millet are less encouraging (Finkelstein et al. 2017). A human study comparing the outcomes when feeding iron-deficient women biofortified to nonbiofortified beans over 128 days showed that the women consuming the biofortified beans consumed more iron and had improvement in the iron status as measured through increased hemoglobin and body iron (Haas et al. 2016). The benefits are most seen in iron-deficient subjects (Finkelstein et al. 2017). Additional studies and approaches to assure success are described in the upcoming sections.

4.5 Iron and Zinc Form and Localization

Iron is stored in both the seed coat and cotyledons, with 2–26% stored in the seed coat, depending on the genotype (Ariza-Nieto et al. 2007; Cvitanich et al. 2010). High levels of iron are concentrated in the outer cotyledon layers surrounding the provascular bundles primarily as ferric iron and in the epidermal layer, mostly in the cytoplasm (Cvitanich et al. 2010). Nonferritin iron levels are highly correlated with total phytic acid levels, suggesting that they are stored together (Hoppler et al. 2014). An estimated 15–29% of iron is stored with ferritin, and ferritin is localized in cotyledon starch granules (Cvitanich et al. 2010). Zinc is found mostly in cotyledons (83–94%) and, in contrast to Fe, is spread more evenly throughout the cotyledons (Cvitanich et al. 2011). Levels of Fe and Zn in the seed coat versus cotyledon are under separate genetic control, and all are quantitative, except for seed coat Fe, which behaves more like a major gene (Blair et al. 2013). These factors may impact retention and bioavailability.

4.6 Fe and Zinc Retention in Cooked Beans

Beans are most commonly eaten as a whole food without fractionation or seed coat removal. The most common preparation method is to soak dry beans in water for 8–12 h and then cook in boiling water until a soft, palatable texture is reached. During the cooking process, soaking water may or may not be discarded prior to consumption depending on the preference of the consumer. Iron retention during soaking and cooking has been reported to range from 67% to 91% depending on genotype (Hummel et al. 2020; Wiesinger et al. 2016), and zinc retention is 66% to 92% depending on genotype (Hummel et al. 2020; Wiesinger et al. 2016). The

genotype-dependent nature of Fe and Zn retention suggests that there is value in screening for genetic diversity for mineral levels in cooked seeds to identify genotypes not only with high raw seed concentrations, but also optimized for high retention values. At least one study looked at genetic variability of cooked seeds in a diversity panel of 245 Andean beans (Andean Diversity Panel) of germplasm especially important in Africa and North America. Cooked seed Fe ranged from 54 to 99 mg/kg and Zn from 21 to 39 mg/kg (Katuuramu et al. 2018). These traits had low narrow-sense heritabilities, however, at 57.4 for Fe and 36.7 for Zn (Katuuramu et al. 2018).

5 Brief Account of Molecular Mapping for Seed Micronutrient Concentration

At least seven Quantitative Trait Loci (QTL) studies have been conducted for seed iron and zinc concentration in beans, including two Andean × Andean: AND696 × G19833 (Cichy et al. 2009), G21242 × G21078 (Blair et al. 2011); two Middle American × Middle American: Shiny Crow × Black Magic (Cichy et al. 2014), G14519 × G4825 (Blair et al. 2010c); two Andean × Middle American populations: DOR364 × G19833 (Blair et al. 2009), BAT93 × Jalo EEP (Freyre et al. 1998; Izquierdo et al. 2018); and one Middle American × wild: Cerinza × G10022 (Blair and Izquierdo 2012). Taken together, these studies were conducted over 16 field trials, and 87 QTL for seed Fe or Zn were identified. These studies were combined for a meta-QTL analysis and 12 meta-QTLs were identified, including 8 that co-localize for Fe and Zn, 2 Fe specific, and 2 Zn specific (Table 10.4) (Izquierdo et al. 2018). Limited work has been done to adapt these QTL for marker-assisted selection, mostly because each one provides only a few mg/kg increase in Fe or Zn that it was not deemed worthwhile to invest in this approach. These QTL may hold promise as fixed variables in genomic selection models to improve prediction accuracies (Izquierdo et al. 2019).

5.1 Association Mapping Studies

Three association mapping studies for seed Fe and Zn have been published to date, including an Andean diversity panel of 206 genotypes (ADP), a European landrace panel of 192 genotypes, and an eight parent advanced generation intercross (MAGIC) population of 437 RILS (Katuuramu et al. 2018; Diaz et al. 2020; Caproni et al. 2020). The results have not been very encouraging for seed Fe, with no QTL detected in either of the diversity panels and only two detected in the MAGIC population (Katuuramu et al. 2018; Diaz et al. 2020; Caproni et al. 2020). The eight parent MAGIC population had one parental line with high Fe and Zn levels. This line, MIB778, derives its high Fe and Zn from an introgression from *P. dumosus* and its pedigree is FEB226/G35575-2P//FEB226, where FEB226 is the source of the introgression from *P. dumosus* (Diaz et al. 2020; Klaedtke et al.

Table 10.4 Meta-QTL and candidate genes for seed iron and zinc in *P. vulgaris*

MQTL	Chr	Trait	Initial number of QTL	Physical position (Mb)		No. genes in MQTL	Candidate genes in MQTL
				Start	End		
QTL_Fe&Zn_1.1	1	Fe–Zn	6	43.3	48.5	553	NRAMP, NA
MQTL_Fe&Zn_2.1	2	Fe–Zn	3	34.5	35	24	
MQTL_Zn_2.2	2	Zn	2	40.5	42.6	216	
MQTL_Fe&Zn_4.1	4	Fe–Zn	2	44.8	46	108	MATE
MQTL_Fe&Zn_6.1	6	Fe–Zn	8	10.2	12.4	69	FRO
MQTL_Fe&Zn_6.2	6	Fe–Zn	8	28.2	29.5	172	
MQTL_Zn_7.1	7	Zn	2	0.1	0.5	42	
MQTL_Fe_7.2	7	Fe	2	29.5	36.9	698	
MQTL_Fe_8.1	8	Fe	2	0.8	3.5	331	
MQTL_Fe&Zn_8.2	8	Fe–Zn	4	12.5	24.4	300	
MQTL_Fe&Zn_9.1	9	Fe–Zn	2	11.7	13.5	160	NRAMP, ZIP
MQTL_Fe&Zn_11.1	11	Fe–Zn	6	2.3	5.3	337	ZIP

Source: Adapted from Izquierdo et al. (2018)

2012). The average linkage disequilibrium (LD) decay in this population was 74 kb. Two seed Fe QTLs were detected, both on Pv06, and one seed Zn on Pv08. All three QTLs were contributed by MIB778 (Diaz et al. 2020). The effect of each of the two Fe QTL was 2.0–2.67 mg/kg addition of iron. Interestingly, both Fe QTL on Pv06 are near meta-QTL for seed Fe and Zn previously reported and based on QTL studies in Andean, Middle American, and inter-gene pool populations (but none in populations with *P. dumosus*) (Izquierdo et al. 2018). In the diversity panels, additional QTL for seed Zn were identified, including one region on Pv01 (European) and Pv07 and Pv10 (ADP) (Katuuramu et al. 2018; Caproni et al. 2020). The limited detection, especially with the diversity panels, is most likely due to the small population sizes used in the studies, in combination with the multiple small effect genes that control seed Fe concentration (Izquierdo et al. 2018).

6 Genomics-Aided Breeding for Biofortification

Numerous genomic resources are available for *P. vulgaris*, making genomic-aided breeding for traits like bioavailability possible (Table 10.5). Currently, there are three sequenced common bean accessions that are being used as reference genomes:

Table 10.5 *P. vulgaris* genomic resources

Database	Reference/source	Resources offered	Last updated
Phaseolus vulgaris Gene Expression Atlas (PvGEA)	O'Rourke et al. (2014)	Gene expression profile of tissues under nitrogen treatments, co-expressed genes, GO and KEGG pathway analysis	Not specified
PhaseolusGenes	University of California, Davis http://phaseolusgenes.bioinformatics.ucdavis.edu/	Markers, QTLs, SSRs, Andean genome (accession G19833) used	2014
Bean Improvement Cooperative (BIC)	http://www.bic.uprm.edu/?page_id=91	Many genetic resources including gene lists and available molecular markers	2020
Legume Federation	https://www.legumefederation.org/en/	Genomic resources for *P. vulgaris* and 19 other legume species	2020
Legume Information System	Gonzales et al. (2005)	Genomic data portal (GDP) for legume species	2020
KnowPulse	Sanderson et al. (2019)	BLAST, JBrowse, genetic markers, visualization tools to help compare varieties, phenotypic data, genetic data for five legume species (*Cicer arientinum*, *Lens culinaris* (in development), *Phaseolus vulgaris*, *Vicia faba*, and *Pisum sativum*)	2019
Phytozome	Goodstein et al. (2011)	2.12-Mb transposon database (Gao et al. 2014), annotations, JBrowse, BLAST	2020

the Andean landrace "G19833" (Schmutz et al. 2014), the Mesoamerican breeding line "BAT93" (Vlasova et al. 2016), and, most recently, the pinto bean UI111 (PI549535) https://phytozomenext.jgi.doe.gov/info/PvulgarisUI111_v1_1. The Andean accession G19833 is an inbred line named Chaucha Chuga that is from Peru. Also, 473 Mb of the 587-Mb genome (~80%) was assembled on 11 chromosome-scaled pseudomolecules with 27,197 protein-coding genes and 31,638 protein-coding transcripts found from Illumina RNA-seq data (Schmutz et al. 2014). The BAT93 Mesoamerican variety was sequenced using a hybrid sequencing strategy where RNA-seq combined with public expressed sequenced tags (ESTs) and cDNA sequences led to the discovery of 30,491 protein-coding genes. Additionally, 2529 small RNAs (sRNAs) were identified from in silico homology modeling and 1033 long non-coding RNAs (lncRNAs) were found from the combination of Arabidopsis thaliana-homology-based predictions and RNA-seq data.

The de novo assembly of G19833 produced Sanger-expressed sequence tags used to rapidly identify gene transcripts. This data was combined 727 million RNA sequencing (RNA-seq) reads from 11 tissues and developmental stages to predict

where expressed genes were in the genome. BLASTP was used to align *Arabidopsis* genes with common bean genes. Genes associated with seed weight and protein synthesis were among the first to be characterized (Schmutz et al. 2014). BLASTP has since been used to identify genes associated with seed iron and zinc accumulation (Izquierdo et al. 2018).

Genome annotation quality continues to improve as the genome sequencing technology improves. The first version of the dry bean genome, Pv v.0.91, was later replaced by Pv v.1.0 and then by Pv v.2.0, which had longer continuous sequences. Homozygous single nucleotide polymorphisms (SNP), homozygous indels, and differing sequences were also corrected with each iteration. The latest version of the genome, Pv v.2.1, combines PacBio long reads with a reannotation of previous genome versions to provide higher-quality and improved accuracy (Lobaton et al. 2018).

6.1 Impact on Germplasm Characterization and Gene Discovery

Andean beans sometimes tend toward higher iron content than Middle American beans (Blair et al. 2010a, b). This may be due to high iron reductase (FRO) activity associated with Andean beans being domesticated in environments with high pH soils that are rich in organic matter and low in iron. Crosses within Andean or Middle American germplasm or between Andean and Middle American lines, have been performed to identify QTL and candidate genes for iron and zinc concentrations. A study on FRO activity in common bean roots using a low × high seed iron cross (DOR364 × G19833) grown in hydroponics with varying concentrations of Fe(III)-EDDHA identified markers for PvFRO orthologs on chromosomes Pv06 and Pv07 based on in silico mapping. Common bean ESTs based on *Arabidopsis thaliana*, *Medicago truncatula*, and *Pisum sativum* FRO1 gene similarity were revealed to be homologous to the FAD and NAD domains at the 3′ end of the FRO gene. From the iron-limited and iron-sufficient growth conditions, two QTLs (Ira2.1 and Ira11.1) were discovered to be located on Pv02 and Pv11, respectively. Ira11.1 is of interest since this QTL is associated with seed iron accumulation but not with the position of the FRO orthologs based on synteny analysis with soybean and *M. truncatula* (Blair et al. 2010b).

The goal of genomics-assisted breeding is to rapidly assay the genetics of a variety and use that information to select desirable traits for breeding populations (Varshney et al. 2009). Current uses of genomics to select for desirable traits such as marker-assisted breeding and selection, which are based on a single or a few QTLs, are expected to be outpaced by high-throughput techniques (Varshney et al. 2005). Instead of a few markers, genomics-assisted breeding would enable many markers to be identified at once.

Next-generation sequencing, genome selection (marker-assisted selection in which genetic markers cover the whole genome), and gene networks have the potential to offer a broader view of the dry bean genome and the genetic pathways that control biofortification (Abberton et al. 2016). Comparative genomics

approaches like those used with *Arabidopsis thaliana* could also be attempted with more closely related species *Medicago truncatula* and *Lotus japonicus* to identify genes associated with nutrient enrichment in legumes.

7 Recent Concepts and Strategies Developed

To meet these biofortification goals, it may eventually be necessary to consider alternative strategies to genomics-assisted breeding such as gene editing, soil–microbe biofortification, and nanotechnology.

7.1 Gene Editing

While there is potential for biofortified transgenic beans to reach consumers worldwide, public perception and lack of incentive are two major barriers for commercialization (Hefferon 2015). Despite precise DNA modification by gene-editing techniques like CRISPR/Cas9, consumers are often hesitant to purchase GMO foods. It is thought that the lack of consumer acceptance is due to perceived lack of naturalness in GM crops, fear of unfamiliar foods, and trust in the food industry (Hefferon 2015; Siegrist and Hartmann 2020). Different information released by both advocates and opponents of biotechnology products adds to the confusion. Some stigma surrounding GM crops come from concerns regarding rigorous risk assessment, monopolies affecting farmers, or misinformation spread by opposition groups. Some of these issues have emerged for dry beans since the first GM common bean was created and commercialized in Brazil (Tollefson 2011).

Brazil is the world's largest bean producer with an average of 3.5 million tons produced annually (approximately 22 million tons is produced globally). The greatest limiting factor in bean production in Latin America is the bean golden mosaic virus (BGMV), a geminivirus transmitted by the whitefly *Bemisia tabaci* that causes heavy yield loss. With no resistant genotypes in the common bean germplasm, the Brazilian Agricultural Research Corporation (EMBRAPA) developed Embrapa 5.1, a BGMV-resistant transgenic pinto bean line, which was then approved by the Brazilian National Technical Commission (CTNBio) in September 2011. The gene editing technology used to produce Embrapa 5.1 was RNA interference (RNAi), a process where the *AC1* viral gene was silenced using double-stranded RNA (dsRNA) (Bonfim et al. 2007; Brod et al. 2013; Lima Aragão 2014).

7.2 Nanotechnology

Nanotechnology is thought to have the potential to affect cellular mechanisms at an atomic scale. Delivery of nutrients to seeds via nanoparticles is currently being explored for use as a fertilizer (Paramo et al. 2020). The goal is to increase vegetative growth and increase nutrient concentration while decreasing risk to humans. Zeolite/

iron(III) oxide has been shown to be less toxic toward humans compared to other fertilizers, and zinc oxide (ZnO) has lowered arsenic and cadmium contents in rice cultures (Jahangirian et al. 2020; Ma et al. 2020). Furthermore, studies show that at least 30 mg/kg of ZnO is required for a significant increase in vegetative growth and mineral content in the leaves and seeds (Salama et al. 2019).

Regardless, nanotechnology is rarely used in plants due to cell toxicity. ZnO, for instance, led to increased homologous recombination events and alleviated transcriptional gene silencing in *Arabidopsis thaliana* at high concentrations (Yang et al. 2018). Nanoencapsulation, which protects the active ingredient and controls its diffusion, interaction, and activity, could be a way to overcome these challenges (Paramo et al. 2020).

8 Genetic Engineering for Enhancement of Micronutrients

Transgenic technology is a pathway to biofortification. The introduction of barley and soybean genes to the wheat genome previously led to iron biofortification in wheat (Masuda et al. 2013). It is possible that introducing nutrient uptake genes into common beans will lead to additional advancements in biofortification status.

8.1 Target Genes

A meta-QTL study has been performed on common beans to identify candidate genes for biofortification. Three of the candidates in the FRO gene family are of particular interest because they have been used to increase the iron content in rice, wheat, and soybean. Many of these genes are involved in both iron and zinc transportation, such as the zinc-regulated, iron-regulated transporter-like proteins (ZIP), multidrug and toxic compound extrusion (MATE), and natural resistance-associated macrophage protein (NRAMP) gene families (Ajeesh Krishna et al. 2020; Astudillo et al. 2013). The ZIP gene family has been well-studied and characterized in common beans. Other than iron (Fe^{2+}), ZIP transporters are also involved in the uptake of several other divalent metal cations, such as manganese (Mn^{2+}), cadmium (Cd^{2+}), and copper (Cu^{2+}). Nineteen PvZIP genes (PvZIP1-PvZIP19) have been identified using QTL analysis so far, with two PvbZIP genes (PvbZIP2 and PvbZIP3) co-localizing at a QTL on chromosome 11 for iron and zinc levels (Ajeesh Krishna et al. 2020; Astudillo et al. 2013). Bioinformatic approaches using publicly available RNA-seq data revealed seven NRAMP genes in common beans involved in mobilizing iron during nodulation and metal homeostasis throughout their growth (Ishida et al. 2018). The NA and MATE gene families have not yet been functionally characterized in common bean.

In rice, transgenic approaches have been more successful than conventional breeding in reaching the iron breeding target of 30% estimated average requirement (13 mg/kg) in polished rice for women and children. Single or combined genes targeted iron uptake, translocation of iron to grain, iron storage in the endosperm,

decreasing antinutrients, and increasing iron bioavailability. Most of the overexpressed genes are found in rice; however, most of the other overexpressed genes for storage or combined approach were from soybean, barley, and *Arabidopsis*. Ferritin, the protein storage form of iron, from soybean (*Soyfer H*-1) and common beans (Pvferritin) has been overexpressed in both rice and wheat (Kumar et al. 2019; Ludwig and Slamet-Loedin 2019).

8.2 Transgenic Approaches in Bean

Transgenes have been introduced to pluripotent bean cells successfully using *A. tumefaciens* and particle gun bombardment (Barraza et al. 2015; Song et al. 2020). Transgenic technology could be used to biofortify crops beyond their current capabilities. Likewise, genes responsible for seed iron biofortification that have already been discovered in species like barley and soybean could be introduced to common beans to increase seed nutrient content. Barriers to using transgenic technologies on dry beans include lack of consumer acceptance and long-term funding. There is currently a negative public perception of transgenic technologies, which directly contradicts the positive perception consumers have toward health foods like dry beans (Beaver and Osorno 2009). Public opinion has not prevented researchers from improving the reproducibility and repeatability of transgenic methods, but whether these results will achieve lasting attention from grant agencies remains to be seen (Song et al. 2020).

9 Nutrient Bioavailability, Enhancement of Promoters, and Reduction of Antinutrients

In order to understand Fe nutrition from beans, one must first recognize the chemical principles of Fe. Iron is one of the most abundant elements on earth, accounting for 5% of the Earth's crust. It is an essential nutrient for virtually all organisms including most bacterial species. In nature, Fe is found primarily in two oxidation states, ferrous (+2) and ferric (+3), with the ferric form being the most common. Iron is a highly reactive element, dissolves readily in most acids, and is insoluble above pH 3 unless complexed by organic acids, proteins, carbohydrates, and other compounds such as phenolic and polyphenolic acids. The ferrous form can be highly soluble above pH 3 when a reducing agent such as ascorbic acid is present in equimolar or greater concentration. For beans, seed coat polyphenols and phytic acid are known to complex Fe, thereby influencing solubility and exchange of Fe with other components of a meal, and also with the intestinal uptake transporter of Fe on the intestinal luminal surface. In short, it is the solubility and exchangeability of Fe with compounds in foods in the physiologically relevant pH range of 2–8 that influences the rate of intestinal Fe uptake.

In the USA, the average daily intake of Fe is in the range of 10–20 mg depending on one's age and dietary habits. Omnivores can ingest a significant amount of their

daily Fe in heme form, approximately 30%, which comes from hemoglobin and myoglobin present in meats (i.e., muscle tissue) and upon consumption of organs such as liver or heart (Pretorius et al. 2016). Novel heme Fe food additives such as leghemoglobin do not represent a significant portion of heme Fe consumption; however, the applications of such forms of heme Fe are expanding rapidly (Fraser et al. 2018). Heme Fe is absorbed by a different mechanism than nonheme Fe and is generally considered to be more bioavailable (Conrad and Umbreit 2002). However, there is a gap in the literature as to how heme Fe is affected by factors such as cooking, degree of digestion of the protein portion of the molecule, and interaction with factors such as polyphenols, ascorbate, and phytate (Cross et al. 2012).

9.1 Iron Uptake and Absorption in the Human Intestine

For humans, Fe is a challenging mineral to absorb. It is only freely soluble at low pH (i.e., less than pH 3). Iron is ingested in milligram amounts daily while in the presence of grams of other food components to which it readily binds with varying degrees of solubility and exchangeability. Thus, to understand Fe bioavailability from foods, one must first have a general knowledge of the physiology of the human gastrointestinal tract.

The process of food digestion starts with mastication (chewing) and the mixing of salivary amylase in the mouth. For the common bean, it is unlikely that salivary amylase provides any significant digestion as much of the carbohydrate in beans is stored inside the cotyledon cells, thus inaccessible to the enzyme, and salivary amylase is inactivated by the low pH of the stomach (Des Gachons and Breslin 2016).

Once food travels down the esophagus and lands in the stomach, several key factors come into play that can determine the bioavailability of Fe present in the meal. First, in healthy individuals with an empty stomach, the pH is low, approximately 1–2 (Gardner et al. 2002). However, once food arrives the pH rapidly rises in proportion to the buffering effect of the meal and mixing of the food begins, along with stimulation of acid secretion and the proteolytic enzyme pepsin. Within minutes, the food starts mixing and begins emptying into the duodenum (Tyssandier et al. 2003). Size of the meal and rate of food intake can also be a factor on the gastric processing of the meal. Once food empties into the duodenum, Fe absorption can begin.

Knowledge of the intestinal mechanisms for Fe uptake and transfer has been well documented (Cegarra et al. 2019; Gulec et al. 2014). The primary site of Fe absorption is believed to be the upper small intestine, primarily the duodenum and perhaps part of the jejunum (Wheby 1970). As food moves into through the upper small intestine, the pH of the luminal contents moves closer to 7 due to neutralization of stomach acid from pancreatic secretions, thus reducing free iron solubility (Gardner et al. 2002). Therefore, bioavailability of Fe depends upon complexation of the Fe by the food or meal ingredients, and the exchange of the bound Fe with the luminal Fe transporter. Expression of the Fe uptake transporter can be found

throughout the intestinal tract, although significantly higher in the upper small intestine (Tako et al. 2008). Despite evidence for the capability for Fe uptake in the lower intestine, many studies, mostly in animal models, demonstrate that very little Fe uptake occurs beyond the small intestine (Patterson et al. 2009). In recent years, studies on the intestinal microbiome indicate that in the lower intestine Fe becomes a "contested nutrient" as Fe is also essential for almost all species of intestinal bacteria (Kortman et al. 2014). Moreover, bacteria have developed multiple mechanisms of Fe uptake in order to meet their daily needs. In general, it appears that Fe uptake in the small intestine begins to decrease as bacteria levels rise in the small intestine (Gorkiewicz and Moschen 2018). Thus, there is strong evidence that Fe uptake does not occur in the lower small intestine and colon simply due to microflora Fe uptake, leaving little to no Fe available for enterocyte uptake. It is important to note that direct studies of this hypothesis have not been reported, presumably due to the invasive nature of a protocol necessary to test the hypothesis in animals or humans. It is therefore a gap in knowledge of Fe uptake factors of humans.

9.2 Iron Intake: Recommended Values and Bioavailability

The term "bioavailability" is most commonly defined as a percent absorbed of the amount ingested. Fractional bioavailability or absorbability would be synonyms for this definition. For individuals at risk of Fe deficiency, the amount of absorbable Fe delivered from a meal is the most meaningful consideration. Thus, high Fe bioavailability from a low Fe concentration meal or low bioavailability from a high Fe concentration can have the same net effect.

A series of dietary reference intake (DRI) values for Fe were developed in 2001 (Russell et al. 2001). These values, which vary by age and gender, include (1) recommended dietary allowance (RDA). The RDA is the average daily level of intake sufficient to meet the nutrient requirements of nearly all (97–98%) healthy individuals. (2) Adequate intake (AI). The AI is the level of intake assumed to ensure nutritional adequacy when evidence is insufficient to develop an RDA. (3) Estimated average requirement (EAR). The EAR is the average daily level of intake estimated to meet the requirements of 50% of healthy individuals. It is commonly used to assess the nutrient intakes of groups of people and to plan nutritionally adequate diets for them. The EAR can also be used to assess the nutrient intakes of individuals. (4) Tolerable upper intake level (UL). The UL is the maximum daily intake unlikely to cause adverse health effects.

Table 10.1 represents the current iron RDAs for nonvegetarians. Distinction is made between vegetarian and nonvegetarian diets as heme Fe present in meat is more bioavailable than nonheme Fe, plus factors present in meat promote the Fe uptake of nonheme Fe in meat, and nonheme Fe from other sources consumed in a meat-containing meal. The RDAs are based on the assumption of a 10% fractional bioavailability value; thus, for an adult woman of reproductive age (14–50 years),

approximately 1.5–1.8 mg of Fe needs to be absorbed daily to meet nutritional needs.

To put these numbers into perspective for bean Fe biofortification, a human efficacy study comparing "normal" versus "biofortified" beans documented measurable nutritional benefit from an estimated increase in daily Fe absorption of 0.27 mg over a 128-day period (Haas et al. 2016). It should be noted that these values come from a population of subjects where 86% were Fe deficient and 37% were exhibiting anemia. In a population at risk of iron deficiency, iron-deficient and -anemic subjects are those most likely to benefit from biofortification. In this Rwandan study, the improvement in Fe status was measureable, yet mild to modest from a physiological perspective.

9.3 Measuring Bean Fe Bioavailability

Assessment of Fe bioavailability from a food or meal in human or animal subjects is a challenging task. In order to track the amount of Fe absorption from food, isotopic labeling has been the most common approach (Fairweather-Tait and Dainty 2002). Isotopic labeling is used for single meal and multiple meal studies and has never been used for long-term or efficacy studies. In recent years, stable isotopes of Fe such as ^{57}Fe and ^{58}Fe are the most commonly used; however, studies have been done with radioisotopes such as ^{59}Fe and utilizing whole-body counting (Hadley et al. 2006). Isotopic labeling of foods can be done via extrinsic or intrinsic labeling. Extrinsic labeling involves the addition of a known amount of isotope to the food or meal, mixing and allowing time (usually 15–30 min) for equilibration with the Fe intrinsic to the meal. Intrinsic labeling requires hydroponic culture where the isotope is added to the nutrient solution and thus incorporated into the plant. The pros and cons of each approach are discussed below.

Extrinsic labeling of Fe in foods was widely utilized for studies of human Fe absorption in the early to mid-1970s. With this approach, a known amount of isotope is added to the known amount of Fe in the food or meal, mixed and allowed to equilibrate for 15–30 min. Various amounts of extrinsic isotopes in studies range from 1% to 100% of the intrinsic Fe, with the most common and recent amount being in the range of 7–30% (Glahn et al. 2015). The extrinsic labeling approach assumes that the extrinsic Fe isotope equilibrates fully with the intrinsic Fe of the food or meal. Absorption of the extrinsic isotope is then measured, and each atom of extrinsic isotope is considered to represent a given number of intrinsic Fe atoms depending on the relative molar amounts. In 1983, a review paper summarized the multiple validation studies of the technique (Consaul and Lee 1983).

Intrinsic labeling of beans is an alternative approach to assess of bean Fe bioavailability that negates the accuracy issues of equilibration associated with extrinsic labeling. This approach requires greenhouse space equipped for hydroponic culture, which limits the amount of material that can be produced. Hydroponic culture is labor intensive, requires large amounts of expensive stable isotope, and results in conditions that are quite different from soil, thus some bean varieties grown

in hydroponics can respond quite differently in terms of yield and seed Fe concentration. Such potential complications should be tested prior to growing beans in hydroponics; however, even beans grown in soil in greenhouse can differ in yield and Fe concentration relative to field-grown conditions. With intrinsic Fe labeling, it is best if the beans are highly enriched with the stable isotope, and this is most easily accomplished by providing only the desired stable isotope in the nutrient solution. Given all of the above, intrinsic labeling is more suited to small-scale studies aimed at delineating mechanisms of Fe uptake or factors that influence Fe uptake from foods as it is simply too expensive to produce more than 1–2 kg of beans for consumption. Production of this amount of material costs approximately $20,000–$30,000 for materials alone, depending on the isotope and hydroponic approach (DellaValle et al. 2015; Donangelo et al. 2003; Tako et al. 2009).

Measurement of Fe bioavailability can also be addressed by in vitro methods. Early in vitro approaches utilized simulated gastric and intestinal of food coupled Fe solubility or Fe dialyzability as an estimate of bioavailability (Miller et al. 1981). However, in vitro studies quickly found that Fe dialyzability is not a consistent measure of bioavailability as Fe can be soluble and tightly bound to compounds and therefore not exchangeable. To address this factor, methodology utilizing a human intestinal cell line evolved, thus adding a living component and enabling detection of Fe uptake (Glahn et al. 1996). The intestinal cells, known as Caco-2 cells since they originate from a human colon carcinoma, have been widely studied and utilized in nutrient uptake studies. In culture, these cells differentiate into enterocytes that function similar to the brush border cells of the small intestine, exhibiting the appropriate transporters and response to factors that influence Fe uptake (Glahn et al. 2002; Martini et al. 2002).

The initial methodology with Caco-2 cells utilized radioisotopes to measure Fe uptake, but was subsequently refined to utilize Caco-2 cell ferritin formation as a relative measure of Fe uptake, thus enabling higher throughput and negating issues of radioisotope handling and equilibration of extrinsic Fe with intrinsic Fe (Glahn et al. 1998a; Yun et al. 2004). The use of ferritin formation also facilitated studies of a broad range of foods, including more complex meals (Pachón et al. 2008a, b). Overall, the combination of simulated (in vitro) digestion coupled with Fe uptake by a human intestinal cell line (Caco-2) provided a more physiological assessment of Fe uptake from foods. This model primarily determines relative differences in Fe bioavailability as Caco-2 cells have been known to fluctuate in responsiveness, yet always maintain relative differences between foods. However, with proper technique and careful attention to detail, the Caco-2 cell ferritin formation response can be highly consistent.

The in vitro digestion/Caco-2 cell model, or more recently known as simply the "Caco-2 Cell Bioassay," has been thoroughly validated relative to human and animal studies (Tako et al. 2016). In addition to the direct parallel comparison of the bioassay to human efficacy trials, numerous publications exist where this model has exhibited qualitatively similar response to those documented in humans (Glahn et al. 2002, 1998a; Engle-Stone et al. 2005). Thus, as an in vitro approach the Caco-2 cell bioassay has attained high credibility as a screening tool for evaluating Fe

nutrition from foods. It has been widely applied to a multitude of foods and food products (Pachón et al. 2008a, b; Tako et al. 2011; Zhu et al. 2009; Beasley et al. 2019; Wortley et al. 2007).

Since inception in 1998, the Caco-2 cell bioassay has advanced the field of Fe nutrition as it filled an essential need to identify important factors not easily defined in vivo and to develop and refine research objectives of the more definitive and costly human studies. It is important to recognize that the Caco-2 cell bioassay measures the relative Fe delivery from a food or meal. In other words, regardless of the amount of Fe in the test meal, it defines the relative amount of Fe that can be taken up at the first level of the absorption process, uptake into the enterocyte. This step is considered the most important in defining Fe bioavailability as most often the goal is to improve or monitor the nutritional quality of Fe in a food. Iron status is regulated by absorption, and iron-deficient individuals upregulate Fe uptake to meet nutritional needs. In the Caco-2 cell bioassay, the standard conditions of the model are designed to have the cells at maximal level of Fe uptake, thus providing a true measure of the potential of the food to deliver Fe.

A number of animal models have been used to measure Fe bioavailability (Brigide et al. 2014; Fairweather-Tait 2001; Welch et al. 2000). Rodents were initially a popular animal model and are occasionally in use today. However, studies have consistently demonstrated that rodents are highly efficient at absorbing Fe from foods that would be low in Fe bioavailability to humans; hence, although the model remains in use today for Fe uptake studies it has fallen out of favor with many investigators. The advantages to rodents are that they are relatively small, thus they do not consume large amounts of diet and are commonly available for research. Rodents can be relatively difficult to handle and are only moderately easy to phlebotomize. They can also be relatively expensive to house and maintain.

Piglets are thought to be an excellent model for studies related to the human intestinal absorption of nutrients (Guilloteau et al. 2010; Patterson et al. 2008). However, unlike humans, piglets are born Fe deficient and require intramuscular injections of 100 mg Fe dextran to help prevent anemia (Hansen et al. 2009). Similar to humans and likely other animal models, investigators have observed that in studies of Fe nutrition it is critical that piglet Fe status be only mildly or borderline deficient; otherwise, upregulation of Fe absorption may negate detection of Fe bioavailability differences in foods (Tako et al. 2009). Piglets can also be challenging to handle due to size and can be selective eaters (Tan et al. 2008). Investigators have also observed that management of diarrhea can be difficult for piglets on experimental diets. Facilities for piglets can also be relatively expensive due to animal size.

As an alternative to rodents and piglets, the poultry model for Fe nutrition has been developed over the past decade (Tako et al. 2010, 2016). This model utilizes the modern broiler chicken and has been well-validated to studies of human Fe nutrition. The broiler chicken possesses many attributes that make it ideal for studies of human Fe nutrition. The animals are relatively easy to handle and phlebotomize, tolerant of a broad range of diets, relatively inexpensive to house, sensitive to changes in Fe

bioavailability, absorb Fe at reasonable amounts, and exhibit strong similarity on a molecular level with human Fe transporters and related genes.

Over the past 5 years, studies have demonstrated that the combination of the Caco-2 cell bioassay coupled with the poultry model can be effective at identifying factors and developing bean varieties capable of delivering more bioavailable Fe. For example, white beans can deliver more Fe than red beans (Tako and Glahn 2010). Components of the diet can enhance the delivery of Fe from beans of higher Fe concentration (Dias et al. 2018). Higher levels of polyphenolic compounds present in black beans can negate the nutritional benefit of higher bean Fe concentration (Tako et al. 2014). The fast cooking trait in beans has been linked to higher Fe delivery in multiple seed types (Wiesinger et al. 2016). Moreover, fast cooking varieties within the yellow bean seed type have been identified that exhibit high Fe delivery (Wiesinger et al. 2018, 2019). Overall, the body of work using the combination of the Caco-2 cell bioassay and poultry model indicates that this approach efficiently addresses research issues in Fe nutrition, with higher throughput and without the caveats associated with isotopic labeling.

9.4 Factors of Bean Fe Bioavailability

For nonheme Fe bioavailability from beans, the traditional primary factors have been phytic acid and seed coat polyphenols (Petry et al. 2015). More recently, the cotyledon cell wall has been identified as a potential major factor as the cotyledon contains most of the Fe in beans, 65–90%, and this cellular structure is not broken down by cooking or the enzymes of the human digestive tract (Glahn et al. 2016). In addition, as more research progresses on bean Fe bioavailability, the trait of cooking also appears to be a factor that can affect Fe bioavailability. Each of these factors will be addressed in detail in the following section.

9.5 Phytic Acid

Phytic acid (PA), also known as phytate, primarily exists as myo-inositol hexaphosphate in the natural unprocessed seed. It is the major storage form of phosphorous in seeds. In beans, phytic acid appears to be primarily located within the cotyledon cells, presumably in the same matrix around the starch granules where most of the seed Fe is located.

Phytic acid has long been known as a factor that inhibits Fe bioavailability from beans and other staple food crops. It has been extensively studied under a multitude of conditions; thus, this section will only address the basic aspect of how PA influences Fe bioavailability. The mechanism of action for this effect is a function of the molar ratio of phytic acid to Fe (PA:Fe), with phytic acid generally being in excess relative to Fe (Glahn et al. 2002; Engle-Stone et al. 2005; Hallberg et al. 1989).

Phytic acid inhibits Fe bioavailability when it is in molar excess greater than a 2:1 phytate to Fe ratio. It does this simply by complexing Fe and thus limiting exchange of the Fe with the luminal uptake transporter. Thus, the greater the phytate concentration, the more chemical advantage it has to complex the Fe. In beans, the PA:Fe is usually 8:1 or higher (Hoppler et al. 2009, 2014). Maximal inhibitory effect seems to occur at 10:1, but this can be modified depending on the presence of uptake promoters such as ascorbic acid (Glahn et al. 2002; Hallberg et al. 1989). Similar inhibitory effects have also been documented for organic acids such as citrate and nitrilotriacetic acid (Glahn et al. 1998b, 2002).

Some studies have suggested that phytate primarily inhibits Fe bioavailability by forming insoluble complexes with Fe; however, phytate-bound Fe has been shown to be highly soluble (Glahn et al. 2002; Engle-Stone et al. 2005; Morris and Ellis 1976). Inhibitory effects on Fe bioavailability due to precipitation are likely due to interactions with other dietary components such as fiber, calcium, and proteins that can become insoluble under certain meal conditions (Schlemmer et al. 2009). Overall, these interactions resulting in claims of loss of solubility in plant-based meals have not been well defined. However, a number of studies have shown that milk proteins and peptides can improve Fe solubility and bioavailability (Yeung et al. 2001). Interactions with other minerals such as Zn, Co, and Mn (Yeung et al. 2005) have been shown to inhibit Fe bioavailability; however, these studies were conducted in the absence of a food matrix, with Fe present as a salt or chelate such as EDTA. Cobalt and Mn only caused inhibition when in great molar excess whereas Zn inhibited Fe uptake at equimolar levels. These results should not be assumed to be similar in the presence of a food matrix as interactions with the matrix can dramatically change the mineral interactions (Gibson et al. 2018; Glahn et al. 2017).

9.6 Seed Coat Polyphenols

Polyphenolic compounds have long been known to influence Fe bioavailability. Polyphenols are present in a wide variety of foods, notably in tea where catechins such as epigallocatechin-3-galate (EGCG) are the predominant form and have been linked to prevention of cancer, diabetes, and cardiovascular and neurological diseases (Khan and Mukhtar 2019). Polyphenols are also present in many staple food crops, such as cocoa, wheat, potato, sorghum, and in pulse crops such as lentils and beans. In the common bean, polyphenols have been extensively studied, and similarly to tea, are linked to multiple health benefits (Ganesan and Xu 2017). However, in regards to Fe nutrition, all polyphenols were thought to be inhibitors of Fe bioavailability (Sandberg 2007). This viewpoint persisted until about 2015 when a study utilizing human intestinal cell (Caco-2) monolayers demonstrated that not all polyphenols inhibit Fe uptake and some actually promote (Hart et al. 2017). Compounds such as kaempferol, kaempferol 3-glucoside, catechin, and 3,4-dihydroxybenzoic acid were identified as Fe uptake promoters. Conversely, quercetin, myricetin, and myricetin 3-glucoside were clearly identified as inhibitors. This study is noteworthy as it was the first to identify the effects of specific

Table 10.6 Seed coat polyphenols in dry beans known to influence iron bioavailability[a]

Polyphenol	Classification	Compound derivatives
Kaempferol	Flavonols	3-*O*-glucoside; 3-*O*-sumbubioside
Quercetin	Flavonols	3-*O*-glucoside; 3-*O*-rutinoside
Myricetin	Flavonols	3-*O*-glucoside; 3-*O*-rhamnoside
Catechin	Flavan-3-ols	(+)-Gallocatechin
Epicatechin	Flavan-3-ols	(+)-Epigallocatechin
Procyanidins	Condensed tannins	Dimers: A2, B1, B2, C1
Cinnamtannins	Condensed tannins	Dimers: A2, B1
Cyanidin	Anthocyanins	3-*O*-glucoside
Delphinidin	Anthocyanins	3-*O*-glucoside; 3-*O*-glucosyl-glucoside
Malvidin	Anthocyanins	3-*O*-glucoside
Pelargonidin	Anthocyanins	3-*O*-glucoside; 3,5-*O*-diglucoside
Petunidin	Anthocyanins	3-*O*-(6″-acetyl-glucoside)

Source: Adapted from Hart et al. (2020), Ganesan and Xu (2017), and Lin et al. (2008)
[a]Polyphenols and their chemical derivatives detected in either Alubia, black, carioca, cranberry, great northern, navy, pink, pinto, red kidney, red mottled, small red or yellow beans

polyphenolic compounds on Fe uptake. Subsequent studies by this same research group characterized a multitude of phenolic and polyphenolic compounds found in beans in regards to their relative effect on Fe uptake (Hart et al. 2017). In addition, this study also demonstrated that the inhibitors of Fe uptake are far more potent in effect than the promoters; presumably due to strong binding of the Fe by the polyphenol. Table 10.6 lists the major promoters of Fe uptake, which are kaempferol, kaempferol 3-glucoside, catechin, and epicatechin; the inhibitors quercetin and quercetin glycosides, myricetin and myricetin glycosides, and various condensed tannins and anthocyanins.

Additional studies by this research group focused on seed coat polyphenols of the yellow bean market class (Hart et al. 2020). Certain varieties of the yellow bean market class have been identified that have high amounts of kaempferol and kaempferol 3-glucoside in the seed coats, with insignificant levels of inhibitor polyphenols present. Overall, the polyphenolic profile of bean seed coats appears to be the strongest factor that defines the bioavailability of Fe from beans in the Caco-2 assay.

In addition to the yellow bean market class, recent research also indicates that the slow darkening trait observed in pinto beans also promotes high Fe bioavailability (Glahn 2019; Wiesinger et al. 2021). The initial studies of normal darkening pintos versus slow darkening varieties indicate that similar to yellow beans seed coat polyphenols such as kaempferol, kaempferol glycosides, epicatechin, and catechin are in high concentration relative to the inhibitor polyphenolic compounds. This observation is significant as the slow darkening trait may be applicable to other market classes and thus represent an approach to improve Fe bioavailability from cranberry, and red and red mottled market classes.

9.7 Cotyledon Cell Wall

Studies have shown that 75–90% of total bean Fe is present in the bean cotyledon (Ariza-Nieto et al. 2007). This intracellular Fe appears to be present in the matrix surrounding the starch granules within the cotyledon cells. A 2016 study demonstrated that cooking and gastrointestinal enzymes do not break down the cotyledon cell wall of beans and other legumes (Glahn et al. 2016). This study agrees with a human study conducted in 1998 that demonstrates the lack of digestion of bean cotyledon cells in the upper and mid small (Noah et al. 1998). In this study, ileal lumen samples demonstrated that bean cotyledon cells only begin to break down after interaction with the intestinal microbiome that populates lower small intestine in significant quantity such that the bacterial enzymes can break down the cotyledon cell walls. It is unknown at present how much of the bean cotyledon Fe is absorbed by humans as Fe absorption has only been shown to occur in the upper small intestine (Wheby 1970). This observation may simply be due to the ability of the intestinal microbiome to outcompete the intestinal brush border cells and absorb any bioavailable Fe present in the intestinal lumen. Indeed, Fe uptake transporters are known to be present throughout the intestinal tract, although at lower density in the more distal sections (Tako et al. 2008) Intestinal bacteria are known to have efficient mechanisms to take up Fe (Gorkiewicz and Moschen 2018). Thus, Fe in the intestinal lumen is now considered to be a "contested nutrient" once it reaches the mid to lower small intestine (Kortman et al. 2014).

Disruption of the cotyledon cell wall via processing has been shown to enhance Fe bioavailability from beans (Glahn et al. 2016). The enhancement of Fe uptake occurs in varieties that have no seed coat polyphenols such as a white bean or a variety in the yellow bean market class that has a predominance of Fe uptake promoting polyphenols in the seed coat. Conversely, disruption of the cotyledon cell wall in varieties with high levels of Fe uptake inhibitors, such as black or red beans, has been shown to decrease Fe uptake relative to uptake from the same varieties with intact cotyledon cells (Wiesinger et al. 2020). This observation indicates that the seed coat polyphenols are complexing the Fe released from the cotyledon cells and inhibiting Fe uptake.

In summary, the cotyledon cell wall has only recently been identified as a significant factor affecting Fe bioavailability from beans and other legumes. Clearly, it is also a factor that could negate the equilibration of extrinsic Fe isotopes in studies that use the extrinsic labeling approach to measure Fe bioavailability from a bean or other legume where the cotyledon cell wall is intact (Petry et al. 2010, 2012, 2013, 2014). Such discrepancy in the extrinsic labeling technique has never been properly evaluated, even after it was critically evaluated 37 years ago (Consaul and Lee 1983), and shown to be flawed in recent studies that revisited the methodology (Glahn et al. 2015; Jin et al. 2008).

9.8 Reduction of Antinutrients (Phytate and Polyphenols)

Reduction of polyphenols in beans is best accomplished simply by consuming beans with a white seed coat. Both in vitro and in vivo studies clearly agree that white beans provide more bioavailable Fe than beans with a colored seed coat (Petry et al. 2010; Tako et al. 2010). The exception to the above is beans of the yellow bean market class, as discussed earlier in this section (Wiesinger et al. 2018; Hart et al. 2020). In this research, some yellow beans have actually been shown to provide equal or more bioavailable Fe. This effect is believed to be a result of an abundance of Fe uptake promoting polyphenols in the seed coat, and little or no inhibitory polyphenols.

Reduction of phytic acid in staple crops has been a widely pursued strategy to enhance Fe bioavailability from staple food crops (Raboy 2002, 2020). In beans, promising studies have been reported on low phytate varieties. The primary concern in reducing phytate is that yield will be low for the crop. However, one study has reported that yield was minimally affected by the lower level of phytate (Campion et al. 2009). These bean varieties were found to have higher Fe bioavailability both in vitro and in vivo (Petry et al. 2013; Campion et al. 2013).

Although lower phytate may enhance Fe absorption from the bean, there is concern that the low phytate crop may have negative effects on human health. One human trial reported that low-phytate beans produced adverse gastrointestinal effects in women (Petry et al. 2016). In addition, one must consider that even though phytate is considered an antinutrient for minerals such as Fe and Zn, phytate is also linked to anticancer benefits in numerous studies (Vucenik 2019). The same can be said for polyphenols (Zhou et al. 2016).

10 Brief Account on Social, Political, and Regulatory Issues

Similar to other new varieties that are released from plant breeding programs, biofortified beans must overcome regulatory, dissemination, and performance hurdles in order for there to be adoption by producers and consumer acceptance. Adoption is one of the greatest challenges that breeders face when developing a new variety, and research to date on adoption of new varieties has identified barriers such as adaptation to the growing environment, suitable agronomic traits for the cropping system (Isaacs et al. 2016; Sperling et al. 2008; Worku 2008), gender- and task-based trait preferences often linked to processing, cooking, and sensory factors, and storability and consumer acceptance (Weltzien et al. 2019). Biofortified breeding programs considered these issues early on by working with interdisciplinary groups of scientists, and they developed targets to meet the specific needs of women and children (Bouis and Saltzman 2016). In a review of biofortified crops, authors found that biofortified crops were acceptable for sensory attributes, and information on the nutritional quality and health benefits improved acceptance and adoption (Talsma et al. 2017). Specific to biofortified beans, a study from Rwanda indicated that farmers preferred a high iron bean variety, for its other attributes, even in the absence

of nutrition information (Birol et al. 2015), and two other studies in Rwanda and Guatemala found that willingness-to-pay (WTP) for biofortified bean varieties did not change based on nutrition information (Waldman et al. 2014). However, Pérez et al. (2018) also found that the type of information and repeat messaging about the iron bean variety in Guatemala was important for WTP. Farmer motivation to grow high iron beans in Rwanda is related to increased yield potential and trying new varieties (Mulambu et al. 2017). Thus, there is evidence that farmers will adopt biofortified bean varieties if they have preferred attributes and they do not cost more than other varieties. Yet, even when varietal attribute conditions are met, an additional hurdle to adoption of new varieties is dissemination and seed supply. Evidence from a recent scoping review of adoption of climate-resilient crops found that access to seed and availability of seed on time, in reasonable proximity, were major barriers to adoption of varieties (Acevedo et al. 2020). Within a conventional seed and variety supply pipeline, dissemination hurdles include production of early generation seed, awareness about the new varieties, delivery, high-risk aversion to unknown varieties, and limited resources for purchasing seed. While biofortified crops offer an important option for alleviating micronutrient deficiencies, and HarvestPlus aims to embed biofortification in the global food system, they still face the same barriers to adoption as other crops in addition to other sociopolitical and regulatory challenges.

Biofortified beans are produced through conventional breeding efforts, but biofortified crops in general have social and regulatory hurdles that are rooted in the first biofortified crop that became widely known 21 years ago, Golden Rice. Golden Rice was generated using genetic engineering, in which two genes from daffodil and one gene from the bacterium *Eriwnia uredovora* were inserted into a variety of rice japonica (Taipei 309) (Sharma et al. 2017). It was purported as a solution to vitamin A deficiency in rice-producing countries, and several varieties were developed to fit environmental and end-user concerns. However, Golden Rice became a flash point for the GM debate (Stokstad 2019), and the residual effects influence perceptions about other conventionally bred biofortified crops. Importantly, the acceptance of GM crops is low across the globe, and the general public does not always distinguish between terms such as "biofortified," transgenic, molecular marker development, conventional breeding, and the more recent new breeding technologies (NTB). There continues to be competing views about the health and environmental ramifications of GM crops: and there continues to be confusion about what conventional breeding and nonconventional breeding entails. This is an underlying concern for potential users of biofortified crops, even though all the released biofortified crops are developed through conventional breeding. The lack of a regulatory definition of biofortified crops may not allay consumer's concerns due to this conflation with GM crops. However, it is also true that conventional breeding is viewed favorably and with appropriate and concentrated messaging to improve awareness and understanding, and biofortified crops have great potential in various markets in both high- and low-income countries (Lockyer et al. 2018).

Conventionally produced biofortified crops, including biofortified beans, have been released all over the world, and 21 governments have incorporated

biofortification into their national health and agricultural plans (https://www.harvestplus.org/what-we-do/engagement2020), but a regulatory framework is not in place. Such a regulatory framework is important for safety concerns, development, approval, and marketing of biofortified crops. Other foods that are fortified with micronutrients have guidelines for testing, labeling, and packaging set out by the WHO and other regulatory entities, but there is not a complementary standard for biofortified crops as a food crop or as ingredients in other foods (Mejia et al. 2017). A global definition of biofortification was finally harmonized and proposed in 2018 by the WHO/FAO-administered *Codex Alimentarius* (Mejia et al. 2017; Codex 2015, 2018), but it remains in question as the proposed draft has limited value for labeling because the terminology is broad (Codex 2019). In the USA, the USDA has defined biofortified as "an increase in the nutritional value of plant foods obtained through conventional crop breeding methods or through crop genetic engineering techniques. This contrasts with postharvest fortification in which nutrients are added during processing" (NAL 2020). In keeping with other labeling and regulatory guidelines in the USA related to GM crops, this definition does not distinguish conventionally bred varieties from GM varieties, which could have ramifications for the acceptance of biofortified crops. In order for biofortified crops to secure the trust of farmers and consumers and expand into markets, a harmonized, global definition is needed, accompanied by appropriate legislation and enforcement, in order to legitimize a regulatory framework. Clear messaging as to the process of developing biofortified crops and food, and appropriate nutrition communication are key to improving their adoption and utilization. But if conventionally bred biofortified crops are combined together with biofortified GM crops in definition and regulations, then they will continue to face adoption and utilization challenges similar to Golden Rice or other GM products, especially outside of the USA.

11 Future Perspectives

Great success has been achieved in developing and implementing the concept biofortification. In beans this includes identifying high iron germplasm sources, increasing seed iron through breeding, developing high iron bean varieties, and promoting them to farmers and consumers. Therefore, with this impressive groundwork that has been laid and infrastructure that has been developed, now is the time to make tweaks and improvements in the process to address some of the challenges and allow for greater reach and societal benefits of biofortification. One challenge with breeding is that not only is iron (and zinc) an invisible trait, it is also highly influenced by the growing environment. Therefore, it is important that evaluation of breeding materials be conducted within the region where they will be grown. It is also important to include local check varieties that are being consumed by the population the crop is intended to feed. This will help ensure that the achieved breeding gains are useful. Going forward it is also important to consider factors other than raw seed iron concentration. The approach of increasing iron concentration has shown modest gains in human efficacy trials. There is a need to support an approach

that focuses on Fe delivery, taking into account both Fe concentration and bioavailability. Iron is an element that is highly interactive with components of the food matrix; thus, simply having more Fe does not always result in more Fe being available for absorption. The combination of factors such as the seed coat polyphenolic profile, the cotyledon cell wall, and the intestinal microbiome requires an approach that measures the amount of Fe that a food or meal delivers for absorption. Therefore, we propose breeding materials be screened for raw seed Fe and cooked seed in vitro Fe bio availability as part of the breeding process. This is essentially the role the Caco-2 bioassay fills for studies of Fe nutrition. It provides a measure of relative Fe bioavailability by measuring the most important step in Fe absorption, uptake from the intestinal lumen into the enterocyte. Such methodology is applicable to the needs of a breeding program with many samples. It is highly cost effective as the Caco-2 bioassay is capable of high throughput and relatively inexpensive, particularly for labs that are set up for cell culture with experienced technicians, and who routinely culture Caco-2 cells. Beyond the in vitro test, it is worthwhile to consider animal feeding trial, with the poultry model, with top breeding lines being considered for release. Moreover, poultry are a less expensive model relative to other animals due to requirements for animal husbandry, growth rates, and age of use. These screening tools represent a data-driven approach to develop products, expose and define mechanisms, and ultimately refine experimental objectives and hypotheses for more definitive and effective human trials.

In addition to iron, it is now time to focus more on zinc. In a recent commentary on biofortification, Steve Beebe indicates that zinc fell away as a target for beans because of lack of progress, but he suggests that it should not be forgotten, especially with new sources of high zinc levels in *P. parvifolius* and new statistics on the prevalence of zinc deficiency in humans (Beebe 2020). Based on an absorption study in young women fed intrinsically and extrinsically labeled high Zn beans (55.4 mg/kg) and normal Zn beans (28 mg/kg), they absorbed 40% more zinc from the high Zn beans (Donangelo et al. 2003).

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Lentil Biofortification

11

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Abstract

Micronutrient deficiencies (MNDs) affect over 2 billion worldwide. Preschool children and pregnant women in developing countries are most affected. Biofortification using conventional and transgenic approaches is a sustainable means to reduce MND. Evaluation of lentil genetic resources has revealed significant variation for micronutrients in both cultivated and wild species. Few biofortified varieties of lentil have been released for cultivation in different countries. The present work comprehensively reviews the efforts being made for lentil biofortification using conventional approaches and molecular tools in which future thrust areas have also been highlighted.

Keywords

Lens culinaris · Micronutrients · Biofortification · Micronutrient deficiency

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1 Introduction

The current global population is 7.6 billion and is estimated to reach 9.8 billion by 2050 (https://www.un.org/). The current exponential population growth rate requires 70% more food to feed this population. The United Nations has taken initiative to end malnutrition and hunger worldwide by 2030 by ratifying sustainable development goals (SDGs) in 2015. The review of progress of sustainable development goals (FAO, IFAD, UNICEF, WFP, and WHO 2019) has revealed that more than 820 million people do not have enough to eat. The global prevalence of undernutrition was 10.8% in 2018. The maximum undernourishment of 19.9% was recorded in Africa followed by 14.7% in South Asia. Moderate to severe level of food insecurity was prevalent in 2 billion people. The situation remains alarming. Nearly 2 billion people suffer from micronutrient deficiencies (MNDs) (Imdad and Bhutta 2012). Micronutrients are essential for human physiology and immunology (Guerrant et al. 2000). Prevention of MND is essential and is being accomplished through biofortification of food crops, supplementation, food fortification, and dietary diversification.

Micronutrients represent mineral and essential vitamins required from diet to sustain normal cellular and molecular functions (West et al. 2012). Essential micronutrients for humans include 11 trace elements and 13 vitamins (Allen et al. 2006; Trumbo et al. 2001). Micronutrient deficiencies are also known as hidden hunger (Stein and Qaim 2007). Micronutrient deficiencies (MNDs) are caused due to lack of dietary diversity. MNDs result in reduced capacity of work, impaired endocrine and immune function, and microcytic anemia. Children below 5 years and pregnant women are at the highest risk of MND. Mediation during pregnancy and first 1000 days is critical (Bailey et al. 2015a). Iron deficiency results in microcytic anemia, and impaired immune and endocrine function. This deficiency is most common and is the highest contributor of maternal death. Zn deficiency causes acute stunting, respiratory infection, and diarrhea, and is one of the causes of death of children below 5 years. Vitamin A deficiency impairs cell differentiation and immune function and causes blindness. Iodine deficiency results in goiter, reduced cognitive function, and mental retardation. Folic acid is required for DNA synthesis and prevention of anemia and neural tube defect. Se deficiency causes cardiomyopathy and osteoarthropathy affecting above 1 billion people suffering from these diseases. Micronutrient deficiencies have detrimental effect on human capital and economic development. Micronutrient deficiencies are reversible if missing micronutrient is provided. However, some disorders are irreversible. Timing, and severity of deficiency determine its after effects. The micronutrient interventions are urgently needed as they are cost-effective interventions to improve global health in low-income and middle-income countries (Global Nutrition Report 2014). The recorded benefits of micronutrient intervention include reduction in low birth weight, increased child survival, and improved cognitive development. Coordinated, multidimensional and sustainable efforts are required to combat MND.

Biofortification is effective in ensuring nutritional security and in decreasing the cost of reducing MND in rural populations of developing countries (Tanyolac et al. 2010).

2 Crop Status

Lentil is among the earliest domesticated crops (Harlan 1992). Lentil is under cultivation for the last 10,000 years in different agroclimatic conditions in different regions of the world. Barulina (1930) made a detailed and complete study of cultivated lentils. She classified cultivated lentil into two subspecies: *Lens culinaris* ssp. *macrosperma* (Baumg. *pro var.*) Barulina and *microsperma* (Baumg. *pro var.*) Barulina. Ferguson et al. (2000), based on morphological and molecular markers, classified genus Lens in four species: *Lens culinaris* (divided into four subspecies: *culinaris*, *orientalis*, *tomentosus*, and *odemensis*), *Lens ervoides*, *Lens nigricans*, and *L. lamottei*. The progenitor of cultivated lentil is *Lens culinaris* subsp. *orientalis* Bioss.) Ponert. Lentil originated in the near East and Central Asia.

From fertile crescent, lentil spread to Greece, Nile Delta, and along Danube to Central and Western Europe. Lentil was carried by Indo-European invasion through Afghanistan (de Candolle 1882).

Lentil is the key winter season food legume of semiarid tropics cultivated in 59 countries worldwide. Global lentil production has increased from 1.00 million ton in 1968 to 6.33 million ton in 2018 (FAOSTAT 2018). The leading lentil-producing countries include Canada (33.64%), India (25.59%), the USA (6%), and Turkey (5.5%) with contribution of over 70% to the world's lentil production. Lentil improves both human and soil health (by biological nitrogen fixation). Lentil is cultivated in different cropping systems (mono and sequential cropping, mixed or intercropping, relay cropping, and multitier cropping). Lentil is grown as a monocrop in Bundelkhand region and Tal areas of Bihar in India. Sequential cropping is common practice in Australia, Canada, Turkey, the USA, and parts of India. The rotational crops include cereals, oilseeds, and pastures. In India, lentil is intercropped with wheat, mustard, linseed, and sugarcane. Intercropping ensures improved land use efficiency, crop productivity, and monetary returns. Lentil is also grown as a relay crop in paddy fields in Eastern India. To exploit residual moisture, lentil is broadcasted in standing crop of rice. In multitier cropping, lentil is grown with crops of different heights (tall trees planted at wide space) (Mishra et al. 2020).

Lentil, being rich in grain Fe and Zn concentration as compared to other grain legumes and cereal, is a superior staple crop for combating MND (Hemalatha et al. 2007; Ray et al. 2014). In particular, lentil is a rich source of micro- and macronutrients and trace elements (Wang and Daun 2006). Lentil grains (whole or split) are consumed as soup or dal. Fried snacks made from lentil whole grains are very popular in India. Pulses with rice are common staple food in South Asia. In the Mediterranean region, lentil is used for making muchaddra. Developed countries have also recognized the nutritional value of lentil and have developed biofortified packed food products like "Crunchy Lentil Chips," "Plentils," "Amy's Organic 74 Soups," "Red Lentil Veggie Soup," "Lentil Crakers," "Barley–Lentils–Risotto

with Avocado 75 Mousse," and pasta, snacks, pizza crust, and crackers (Kumar et al. 2016). The high nutritional value of lentil makes this crop ideal for biofortification. The crop is popular in both developing and developed countries.

3 Nutritional Value

Lentil is a rich source of protein, macro- and micronutrients, carbohydrates, and phytochemicals (Dueñas et al. 2002; Rochfort and Panozzo 2007; Thavarajah et al. 2011b). Grusak (2009) reviewed the nutritional quality of lentil and reported protein content in the range of 15.9–31.4%, carbohydrate 43.4–74.9%, fat 0.3–3.5%, total fiber 5.1–26.6%, and ash 2.2–6.4%. Thavarajah et al. (2011b) reported that consumption of 100 g lentil grain provides 41–113% of the recommended daily allowance (RDA) of Fe, 40–68% of Zn, 77–122% of Se, and 2–12 μg/g of beta-carotene. The storage protein is located in cotyledons. Lentil is rich in total soluble fiber (Brummer et al. 2015). Lentil protein comprises 70% globulins, 16% albumins, 11% glutelins, and 3% prolamins (Boye et al. 2010). Lentils are rich in all essential amino acids except sulfur-containing methionine and cysteine. Lentil is an excellent complimentary food to cereals. Lentil is rich in lysine and cereals in sulfur-containing amino acids (Shewry and Halford 2002). Lentils are low in mono- and disaccharides and oligosaccharides. Oligosaccharides are metabolized in large intestine by colon bacteria-releasing gases (hydrogen, carbon dioxide, and methane). Starch is the main polysaccharide in lentil. Starch is a key source of energy and is composed of amylose (20–45.5%) (Urbano et al. 2000) and amylopectin. Amylose is digested by pancreatic α- and intestinal sucrose-isomaltase and maltase-glucoamylase (Nichols et al. 2003) in small intestine-releasing energy. Starch, when not digested, known as "resistant starch," passes into large intestine, causing flatulence. Resistant starch value of 3.7 g/100 g dry matter of lentil was reported by de Almeida Costa et al. (2006). Lentil starch is digested, slowly releasing glucose. Lentil has the low glycemic index among food crops (Jenkins et al. 2012). García-Alonso et al. (1998) reported a glycemic index of 42–50 in lentil. Lentil is low in fats (0.3–3.5 g/100 g).

Lentil contains a high proportion of essential fatty acids such as linoleic and linolenic. Nikolić et al. (2013) compared fatty acids and acylglycerol of lentil and chickpea. It was found that in lentil flour lipid content was 1/3 (-0.92 ± 0.04 g/100 g) of chickpea (3.11 ± 0.19 g/100 g). Myristic, heneicosanoic, and eicosatrienoic acids not found in chickpea were reported in lentil, and palmitic acid was reported only in chickpea. Lentils are rich sources of both macro- and micronutrients. Lentils possess both water- and fat-soluble vitamins. Lentil is a good source of folates. Phytic acid (myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is in storage form of phosphorus and is 1–5% by weight in legumes, oilseeds, cereals, and nuts (Vats and Banerjee 2004). During ripening period, phytate accumulates in seed. It is stored as globoid crystal within protein bodies in pulses (Erdman 1979). Using Caco-2 cell model, it was reported that Fe uptake from lentils is relatively greater than other staple food crops due to low PA-phosphorus

(Thavarajah et al. 2011b). Lentil reduces colon cancer and type −2 diabetes and is also involved in cholesterol- and lipid-lowering effect. Adebamowo et al. (2005) have reported that dietary flavonol in lentil lowers the incidence of breast cancer. Phytochemicals like phenolic acid, saponins, flavonols, condensed tannins, and phytic acid were initially considered as antinutritional factors due to their beneficial role to human health being reported (Xu and Chang 2010). Phytochemicals reduce the risk of chronic diseases including neural disorders (Parkinson's and Alzheimer's disease), cancer, cardiovascular diseases, and diabetes. Lutein reduces the incidence of macular degeneration, cancer, and cataracts (Olmedilla et al. 2001). Carotenoids and tocopherols act as antioxidants, promoting skin and eye health. Antioxidant activities of phenolics and lipophilic compounds have been reported by several workers (Dueñas et al. 2002; Xu and Chang 2010).

4 Nutritional Traits for Biofortification

4.1 Protein

The word protein is derived from the Greek word "proteios" meaning primary. Protein is the basic component of human tissue (Wu 2013). Protein comprises 20 different amino acids linked by peptide bonds. Dietary protein is disintegrated by proteases and peptidases into amino acid dipeptides or tripeptides in small intestine. Relative proportion of amino acids content, and digestibility coefficients determines the nutritional value (Reeds et al. 2000). Amino acids are indispensable for development, health, growth, lactation, reproduction, and survival of organism. Amino acids bestow nitrogen, sulfur, and hydrocarbon skeletons. Amino acids are important for synthesis of DNA, RNA, peptides, proteins, creatine, glutathione, dopamine, nitric oxide, and serotonin (Gabriel and Uneyama 2013; Wu 2009). During absorptive state in small intestine, aspartate, glutamine, and glutamate act as metabolic fuel. Glutamine is a source of energy for arteries in postabsorptive stage (Reeds et al. 2000). Glutamine sustains immune response by providing ATP to macrophages and lymphocytes (Wu 2009). In developing countries, protein deficiency causes kwashiorkor and marasmus (protein and energy deficiency). Overconsumption of dietary protein can cause hepatic or renal dysfunction.

4.2 Micronutrients

Micronutrients include essential minerals and vitamins required for molecular and cellular functions in human body (West et al. 2012). Inadequate intake (under nutrition) of micronutrients causes "hidden hunger." Undernutrition is due to food insecurity and inadequate feeding practice and access to health services. Poverty is the main cause of undernutrition. Iron is necessary for cellular respiration and oxygen transport. Fe is an important constituent of cytochromes, hemoglobin, enzymes, and myoglobin. The bioavailability of heme iron is 12–25% and nonheme

iron is less than 5% (Institute of Medicine (US) Panel on Micronutrients 2001). Hemoglobin concentration, plasma ferritin, and transferring saturation are used to assess iron status in human body. Ferritin indicates Fe storage in human body and hemoglobin determines anemia. Globally around 1.62 billion people suffer from anemia (de Benoist et al. 2008a). Pregnant women and preschool children are most susceptible to anemia in low-income countries. Zinc is important for DNA and protein synthesis, cell division, and immune system function. It plays a role in cellular metabolism and is required for the activity of more than 200 enzymes. Zinc deficiency causes stunting and has been linked to diarrhea and malarial infection (Black et al. 2013). Zinc deficiency is the main reason for morbidity in the developing world (de Benoist et al. 2007). Selenium is vital for amino acids, enzymes, hormones, selenoproteins, and antioxidants (Rayman 2002). Most soil is low in Se (100–2000 μg/kg) (Swaine 1955). Over 1 billion people suffer from Se deficiency (Lyons et al. 2003). Selenium deficiency can cause Keshan disease and Kashin–Beck disease (Reilly 1996), immune deficiency, heart disease and thyroid problem (Arthur and Beckett 1994), and male infertility (Behne et al. 1997). A 100 g serving of lentil ensures 77–122% RDA of selenium (Thavarajah et al. 2008).

Fat-soluble vitamin A is necessary for vision, cell differentiation, reproduction, immune function, and organ formation and growth. The plant source of vitamin A is provitamin A, and retinols and retinyl esters are the animal source. Deficiency of vitamin A reduces immunity, causing morbidity and mortality (Sommer et al. 1983) and blindness (West et al. 2012). Vitamin A deficiency is very common during pregnancy in the developing countries. Serum retinol concentration of below 0.70 μmol/l is an indicator of vitamin A deficiency. Iodine is required for synthesis of thyroid hormone (essential for human growth and development). During pregnancy, iodine requirement is increased due to increased renal clearance and metabolism (Glinoer 1997). Maternal iodine deficiency in pregnancy can cause neurological problems and mental retardation (Zimmermann 2009). Iodine deficiency causes goiters, hypo- or hyperthyroidism, and impaired mental function. Iodine status of human body is assessed via urinary iodine concentration. Urinary iodine below 100 μg/l is considered as insufficient and indicates deficiency. Nearly 2 billion people suffer from iodine deficiency (de Benoist et al. 2008b)

4.3 Folates/Vitamin B9

Folates occur as tetrahydrofolate and as polyglutamates in the presence of glutamate residues (Bailey and Caudill 2012). Folates are water soluble and are not retained in human body; their regular intake is required. Folates act as coenzyme in the synthesis of RNA and DNA and is involved in amino acid metabolism (Bailey and Caudill 2012; Ziegler 2012). Folates play a vital role in conversion of homocysteine to methionine in the synthesis of *S*-adenosyl-methionine, an important methyl donor. Methylation of deoxyuridylate to thymidylate is regulated by folates during the cell division. Folate deficiency causes megaloblastic anemia (Carmel 2005), neural tube defect (Geisel 2003), cardiovascular disease (MCCully 2007), and impaired

cognitive function (Ramos et al. 2005). In human gut, folates are disintegrated into monoglutamate and absorbed by intestinal mucosa. Enzyme dihydrofolate reductase reduces the monoglutamate to tetrahydrofolate, converting it to methyl or formyl form (Bailey and Caudill 2012). The folate status in human body is assessed by serum folate concentration (value above 3 ng/ml is considered as adequate) (Bailey et al. 2015b). Staple crops such as rice, potato, maize, and plantain are low in folate (USDA-ARS 2012). The recommended RDA of folates is 600 for pregnant women and 400 mg for adults (Institute of Medicine, Food and Nutrition Board 1998). A 100 g lentil serving can provide 54–73% of folate RDA (Thavarajah et al. 2008)

4.4 Carbohydrates (Prebiotic)

Prebiotic carbohydrates are complex carbohydrates having low digestibility in the upper part of gastrointestinal tract and are fermented by intestinal microbiota stimulating growth and activity of health-promoting bacteria (Oku and Nakamura 2003). Whole grains are rich in prebiotic carbohydrates. Food low in prebiotic carbohydrate increases the risk of obesity and noncommunicable diseases. Lentil is consumed as whole grain with minimal processing and is rich in prebiotic carbohydrates (Bhatty 1988; Johnson et al. 2013). Prebiotic carbohydrates transform microbial composition in gut. Fatty acids are produced and intestinal movement is regulated. Diets rich in prebiotic carbohydrates increase mineral absorption, regulating cholesterol and glucose levels (Kaur and Gupta 2002).

4.5 Phytic Acid

Phytic acid chelates micronutrients, reducing their bioavailability in monogastric animals including human beings lacking phytase enzyme in their digestive tract (Boling et al. 2000; Singh et al. 2011). About 70% phosphorus of total P feed is excreted by monogastric animals (Jorquera et al. 2008). Phosphorus through leaching causes eutrophication of surface water, causing algal blooms, death of fish and aquatic animals, hypoxia, greenhouse gas, and nitrous oxide (Mallin 2000). During seed germination, phytate are reduced by phytase and utilized as phosphate and inositol (Asada et al. 1969).

4.6 Phenols

Phenol-rich diets provide protection against osteoporosis, type 2 diabetes, neurodegenerative diseases, cancer, cardiovascular diseases, pancreatitis, lung damage, and gastrointestinal problems (Fraga et al. 2010; Martin-Pelaez et al. 2013; Fujiki et al. 2015; Xiao and Hogger 2015). Phenols are scavengers of free radicals (Sroka and Cisowski 2003). Phenols act as antioxidants protecting against oxidative stress by producing hydrogen peroxide (Sroka and Cisowski 2003; Saeidnia and Abdollahi

2013). Phenolic compounds chelate metals, reducing their bioavailability (Kulbat 2016). In the recent years, plant-based phenols have been identified as a safe source of antioxidants and these are alternative to synthetic antioxidants.

4.7 Fatty Acids

Unsaturated fatty acids like linolenic, oleic, and linoleic are the major components of fatty acid profile along with small amounts of palmitic acid (saturated fatty acid).

4.8 Dietary Fibers

Dietary fibers are non-nutrients comprising raffinose—family oligosaccharides, polysaccharides, and resistant starch. Dietary fibers play an important role in facial water balance and facial movement in gastrointestinal tract. Part of fiber is fermented in large intestine, releasing energy and promoting microflora.

5 Biofortification

Biofortification refers to the increase in nutrition density in crops by plant breeding, management, and modern biotechnology (Nestel et al. 2006; Chen et al. 2009). Agronomic biofortification improves nutritional quality of a crop. It is a complimentary approach for developing biofortified crops with improved micronutrient concentration. Agronomic biofortification can be carried out through basal application, foliar application, or seed treatment of micronutrients. Duxbury (2005) reported that seed priming of lentil with zinc increased seed zinc concentration. Basal application of micronutrients involves the addition of inorganic substances to soil. The phytoavailability of minerals is low in soil; therefore, minerals with high solubility and mobility can only be applied to improve their concentration in grains (White and Broadley 2009). Rasheed et al. (2020) applied three doses of zinc (0, 6, and 9 mg/kg) to 16 lentil genotypes and reported differential response of studied genotypes. The response from dose 9 mg/kg was better in comparison to other doses. Foliar application permits effective allocation of nutrients to the edible portion of plants in an effective way (Lawson et al. 2015). Rahman et al. (2015) applied 40 g/ha of selenium as potassium selenate (K_2SeO_4) and reported an increase in Se concentration of lentil seeds by more than 10 times. Foliar application of micronutrients increases the cost of production, and micronutrients applied can be washed away by rains (Garcia-Banuelos et al. 2014).

Genetic biofortification is a cost-effective and sustainable approach utilizing genetic variation (in crop species for micronutrient concentration) for increasing the micronutrient concentration in edible portions of plant (Nestel et al. 2006). This biofortification strategy uses both classical breeding and modern genomic approaches. Lentil is a rainfed crop, and the primary breeding objective is increase

of productivity. The increase of productivity has been achieved by hybridization of Indian and Mediterranean germplasm, selection for biotic and abiotic stresses, increased biomass and harvest index, and reduced maturity duration. Further yield increase is required for food security. Improvement of micronutrient concentration and other nutritional quality parameters is gaining importance. With the development of genomic resources in lentil, genomic-assisted breeding can complement conventional breeding for the development of micronutrient-rich lentils. The advancement in genotyping technologies and high-throughput screening has cut effort, cost, and time. NGS technologies are facilitating genome sequencing in lentil. Marker trait associations (MTA) can be utilized for marker-assisted breeding.

5.1 Germplasm Screening

Screening of existing natural variation for target nutritional traits is the basic step for identification of genetic variability for utilization in breeding programs. Substantial genetic diversity for grain Fe, Zn, selenium, folates, and β-carotene has been reported by different researchers (Table 11.1). While screening for grain micronutrient concentration efforts should be made to ensure that genetic resources are grown in homogenized soil. Soil homogeneity or uniformity is necessary for proper screening. Lentil germplasm has been evaluated for grain Fe and Zn concentration by several workers (Kumar et al. 2014, 2019; Ray et al. 2014; Khazaei et al. 2017; Singh et al. 2017; Vandemark et al. 2018). Sarker et al. (2007) evaluated 1600 accessions of land races, wild types, and breeding lines. Lentils are rich in organic Se, selenomethionine (Thavarajah et al. 2008). Variation for Se concentration in lentil germplasm has been reported (Thavarajah et al. 2008; Ray et al. 2014; Vandemark et al. 2018).

Han and Tyler (2003) determined folate concentrations in pulses by a microbiological method employing trienzyme extraction and reported folate concentration range of 151–479 μg/100 g in lentil. Rychlik et al. (2007) compared folates in different food legumes and reported range of 110–154 μg/100 g for lentil. Hefni et al. (2010) compared folate concentration of food commonly consumed in Egypt and reported folate concentration of 75 μg/100 g in lentil. Sen Gupta et al. (2013) compared folate range of legumes and found that lentil has higher folate concentration as compared to fieldpea and chickpea and reported folate range of 216–290 μg/100 g in lentil. Jha et al. (2015) compared folate range of lentil, chickpea, common bean, and pea cultivars grown in Canada and reported range of 136–182 μg/100 g in lentil cultivars CDC Maxim, CDC QG-11, CDC SB-2, and CDC Greenstar (evaluation at Limerick and Sakatoon). Zhang et al. (2018) reported folate concentration of 161 μg/100 g in CDC Maxim and 115 μg/100 g in CDC Greenstar. Folate concentration in *Lens* species was investigated by Zhang et al. (2019) using ultra performance liquid chromatography and mass spectrometry (UPLS-MS). Wild species exhibited higher folate concentration in comparison to the cultivated species. *Lens tomentosus* exhibited median values of 439.7 and 360.9 μg/100 g under field and glasshouse conditions, respectively. It was followed by *Lens orientalis* with median

Table 11.1 Variation for grain Fe and Zn concentration documented in various studies

Micronutrient	Germplasm/population	Concentration range	Reference
Iron (mg/kg seed)	Land races, wild types and breeding lines	41–132	Sarker et al. (2007)
	Germplasm	50.85–136.91	Kumar et al. (2014)
	Germplasm	76–100	Ray et al. (2014)
	Germplasm	41–102	Khazaei et al. (2017)
	Germplasm	31.55–119.35	Singh et al. (2017)
	Germplasm	31.55–119.35	Singh et al. (2017)
	Germplasm	69–86	Vandemark et al. (2018)
	Germplasm	42.8–110.63	Kumar et al. (2019)
Zinc (mg/kg seed)	Germplasm	27–77	Sarker et al. (2007)
	Germplasm	40.2–80.57	Kumar et al. (2014)
	Germplasm	23–54	Khazaei et al. (2017)
	Germplasm	22.08–73.92	Singh et al. (2017)
	Germplasm	27.8–75.45	Singh et al. (2017)
	Germplasm	46–55	Vandemark et al. (2018)
	Germplasm	38.18–81.68	Kumar et al. (2019)
Selenium	Germplasm	425 and 673 μg/kg	Thavarajah et al. (2008)
	Germplasm	636–868 ng/g	Ray et al. (2014)
	Germplasm	0.38–0.52 μg/g	Vandemark et al. (2018)
Folates	Germplasm	1151–479 μg/100 g	Han and Tyler (2003)
	Germplasm	110–154 μg/100 g	Rychlik et al. (2007)
	Germplasm	75 μg/100 g	Hefni et al. (2010)
	Germplasm	216 to 290 μg/100 g	Sen Gupta et al. (2013)
	Germplasm	137–182 μg/100 g	Jha et al. (2015)
	Varieties	115–161 μg/100 g	Zhang et al. (2018)
	Wild species	1.7–5.0	Zhang et al. (2019)
β-Carotene	Cultivars	2–12 μg/100 g	Thavarajah et al. (2011b)
Phytic acid	Cultivars	2.5–4.4 mg/g	Thavarajah et al. (2011a)

values of 416.6 and 327.6 μg/100 g under field and glasshouse conditions, respectively. The range of 2–12 μg/100 g for 2–12 μg/100 g β-carotene in lentil was reported by Thavarajah et al. (2011b).

Wild species are a rich source of gene(s) lacking in cultivated gene pool (Tanksley and McCouch 1997). Significant efforts were made to collect and conserve wild relatives of legumes (Plucknett et al. 1987; FAO 1996). The International Centre for Agricultural research in the Dry Areas (ICARDA) has collected and maintained 587 accessions of different *Lens* species collected from 26 countries. The collected accessions include *Lens culinaris* ssp. *orientalis*, *Lens culinaris* ssp. *odemensis, Lens nigricans, Lens ervoides*, and *Lens lamotti.* Among these, *Lens culinaris* ssp. *orientalis* and *Lens culinaris* ssp. *odemensis* are crossable with the cultivated lentil (Fratini et al. 2004; Fratini and Ruiz 2006; Muehlbauer et al. 2006). *Lens nigricans* and *Lens ervoides* are not crossable with cultivated lentil (Gupta and Sharma 2005) due to hybrid embryo breakdown. Interspecific crosses often exhibit embryo abortion, albino seedlings, and hybrid sterility (Gupta and Sharma 2005; Ladizinsky 1993). Tissue culture techniques like embryo rescue can facilitate in alien gene introgression from related species (Tullu et al. 2013).

The *Lens* wild relatives are an important source of genes for traits of interest (Singh et al. 2014). Prebreeding is a step forward for introgression of alien genes for biofortification of lentil. Limited efforts have been made in lentil for the evaluation of wild relatives for nutritional quality traits. Kumar et al. (2016) evaluated 10 accession of wild *Lens* and reported variation for protein content, phenol concentration, and antioxidant activity. Kumar et al. (2018) have evaluated the biofortification potential of *Lens* species. Core set of 96 accessions derived from 405 global wild annual collection comprising different *Lens* species was examined. Grain Fe concentration ranged from 28.2 to 141.2 mg/kg seed, and grain Zn concentration ranged from 12.9 to 126.2 mg/kg seed. The maximum variability for grain Fe concentration was recorded in *Lens culinaris* ssp. *Odemensis*, and accessions ILWL 243 and EC 718311 were found most promising with 141.2 mg/kg grain Fe. For Zn, *Lens culinaris* spp. *orientalis* was found to be most promising (ILWL 117 exhibited maximum grain Zn concentration of 124.6 mg/kg seed). The Indian Agricultural Research Institute also evaluated limited number of *Lens* accession, and the variation recorded is presented as Fig. 11.1. The range of genetic variation is higher in wild species compared to cultivated lentil. The limitation of use of wild types for Fe and Zn enrichment is due to the fact that mineral concentration is reduced as yield increases in cultivated types due to the dilution effect (Ortiz-Monasterio et al. 2007).

5.2 G × E Interactions

Thavarajah et al. (2008) evaluated 19 lentil genotypes for grain Fe and Zn concentration and reported range of 73–90 mg/kg for Fe and 44–54-mg/kg for Zn. Broad-sense heritability of 64% was reported for grain Fe and 68% for grain Zn concentration. Karaköy et al. (2012) evaluated 39 landraces and 7 cultivars. Zn concentration correlated with other minerals, indicating similar pathways or transporter controlling the uptake and transport of these minerals. Some genotypes

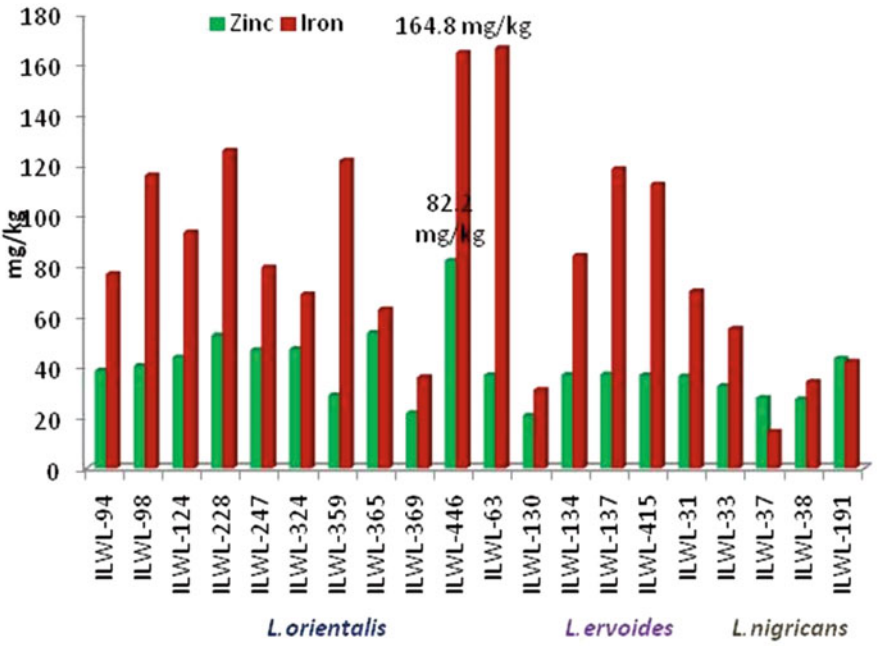

Fig. 11.1 Evaluation of *Lens* species for grain Fe and Zn concentration. (Source: ICAR-IARI, New Delhi, unpublished data)

exhibited high mineral and protein content, indicating potential to improve the nutritional value of lentil. It was suggested that cross-breeding of Turkish land races could improve macro- and micronutrient profile of lentil. Kumar et al. (2014) studied 41 lentil genotypes over three locations and reported high Fe G × E interaction in comparison to Zn. Significant effect for genotypes, locations, and genotype × location was recorded. Singh et al. (2017) studied 96 lentil genotypes at three locations and reported highly significant variation attributed to genotype, environment, and genotype and environment interactions for both Fe and Zn concentration. Using Eberhart and Russell model, it was reported that P 2124, P 2126, and P 2127 were stable for grain Fe concentration and NDL 11-1 and L 4648 were stable for grain Zn concentration.

Thavarajah et al. (2008) evaluated 19 lentil genotypes for Se concentration at 10 locations for 2 years in Canada and reported significant genotype, years, location, and interactions between year × genotype and location × genotype for Se concentration. Rahman et al. (2013) evaluated seven advanced breeding lines at four locations. These were evaluated at farmers' field and in yield trials. Significant genotype and location × genotype effects were recorded. Year × location effects were not significant in this study. Ates et al. (2016) evaluated 96 RILs from cross PI 320937 × Eston in three environments during 2012 and 2013 for grain Se

concentration and reported highly significant genotype, location, year effects, and the interactions between year × genotype and location × genotype.

Sen Gupta et al. (2013) evaluated 10 lentil genotypes at three locations in the USA and reported significant year × location interaction on lentil folate concentration. Jha et al. (2015) evaluated four lentil cultivars using ultra performance liquid chromatography coupled with mass spectrometry for folate concentration and reported range of 136–182 μg/100 g. G × E studies revealed significant differences among the cultivars and significant location and cultivar × location effects.

Thavarajah et al. (2009) reported low phytic acid in lentil. Phytic acid is antinutrient in legumes and cereals. Phytic acid binds with mineral micronutrients, reducing their bioavailability. Nineteen genotypes were evaluated at two locations, and phytic acid range of 2.5–4.4 mg/g was reported. Decortication before cooking reduced total phytic acid by >50%. The lowering of phytic acid intake increases mineral bioavailability.

5.3 Breeding Targets and Target Population

Breeding targets for grain Fe and Zn concentration are based on estimated average requirement, daily intake, and bioavailability. The breeding target for Fe grain concentration in lentil is 70 ppm, and for Zn the breeding target is 50 ppm. The breeding target is 30 and 15 ppm above the baseline for grain Fe and grain Zn concentrations, respectively. Within available gene pool, sufficient variation exists (Table 11.1) for improvement of grain Fe and Zn concentration. Promising genetics variations for selenium and folates have been highlighted in Table 11.1.

5.4 Breeding Strategies

The primary objective of lentil breeding program is to enhance the productivity by selecting for tolerance/resistance for biotic and abiotic stresses, increasing biomass and harvest index, and reducing the maturity duration. Higher lentil production is required to cater to the nutritional demands of growing population. During recent years, nutritional quality and specifically micronutrients have received the attention of lentil breeders. Micronutrient estimation studies have highlighted genotypic variation available in lentil for micronutrient concentration.

Biofortification challenge program (BCP) was approved by CGIAR in November 2002 with funding support from the World Bank and Bill and Melinda Gates Foundation. By mid-2003, BCP was renamed as HarvestPlus. In the first phase of HarvestPlus (2003–2008), defined as Discovery phase, the emphasis was on the identification of target populations and assessment of feasibility of biofortification. In the second phase (2009–2013), the emphasis was on development and release of biofortified crop varieties and their nutritional efficacy trials. In this phase, lentil biofortification program was started under the leadership of ICARDA for the development of iron- and zinc-rich varieties with high yield potential. The initial

Table 11.2 Lentil biofortified varieties released for commercial cultivation

Country	Variety	Fe (mg/kg seed)	Zn (mg/kg seed)
Bangladesh	Barimasur-4	82.6	
	Barimasur-5	86	59
	Barimasur-6	86	63
	Barimasur-7	81	
Nepal	Sisir	64	
	Khajura-2	100.7	59
	Khajura-1	58	
	Sital	59	
	Shekhar	83.4	
	Simal	81.6	
India	L 4147	78	
	L 4717	65	
	IPL 220	112	
Syria/Lebanon	Idlib 2	73	
	Idlib 3	72	
Ethiopia	Alemaya	82	66

focus was on the identification of Fe- and Zn-rich lentil varieties and their upscaling. ICARDA, in collaboration with National programs of India, Nepal, Bangladesh, and Ethiopia, took the lead in exploiting the genetic variation for micronutrient concentration in lentil. The collaborative effort has resulted in the development of high-yield disease-resistant and early-maturing varieties of lentil rich in grain micronutrient concentration (Table 11.2).

In lentil, grain Fe and Zn concentration is reported as quantitative trait (Diapari et al. 2015; Aldemir et al. 2017). The studies have revealed positive correlation between grain Fe and Zn concentration, revealing the similarity in physiological and genetic factors controlling them. The breeding strategies are focusing on the transfer of genes governing grain Fe and Zn concentration from land races and diverse germplasm to cultivated lentils. Efforts are being made to combine high grain Zn concentration in Mediterranean germplasm with iron-rich South Asian germplasm. ICARDA constitutes LIEN MN nursery every year for global partners to select the suitable material. At the Indian Agricultural Research Institute, New Delhi, Indian and Mediterranean germplasm/landraces and breeding lines were screened to estimate grain Fe and Zn concentration and study G $\times$ E interactions for these traits. SSR markers linked to these traits have been identified through association mapping approach (Singh et al. 2017; Kumar et al. 2019). Biparental mapping population is being developed to validate these markers.

5.5 High-Throughput Screening Methodology

Different methods are being used for the estimation of grain micronutrient concentration in different crops. Accurate, inexpensive, and fast methods are required for evaluating a large number of genotypes for selection of genotypes with high micronutrient concentration. Routinely inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma-optical emission spectrometry (ICP-OES) are used for micronutrient estimation. These methods require sample preparation, costly contamination-free chemicals and equipment, and trained manpower. Due to these reasons, lentil breeders working in remote locations are forced to outsource this activity. Calorimetric approach for the estimation of grain micronutrient concentration is simple but laborious (for large sample size). Paltridge et al. (2012) standardized energy-dispersive X-ray fluorescence spectrometry (EDXRF) for the estimation of grain Fe, Zn, and Se concentration. X-ray fluorescence techniques can phenotype a large number of genotypes for micronutrient concentration. The results of XRF (promising accessions identified) can be confirmed by ICP-MS.

6 Gene Discovery

The knowledge on genetic factors controlling grain micronutrient concentration is essential for marker-assisted selection. QTL analysis is an effective tool for determining these genetic factors. The methodology for QTL mapping in crop plants was proposed by Collard et al. (2005). Using biparental crosses, F2, RILs, NILs, double haploid, and backcross populations can be developed. The progenies of population are phenotyped. Marker(s) linked to trait of interest are identified by bulk segregants analysis suggested by Michelmore et al. (1991). Ates et al. (2016) mapped QTLs for grain selenium concentration using 96 recombinant inbred lines from cross "PI 320937" × "Eston." The RILs were evaluated in three environments over 2 years. The range of Se concentration in RILs was 119–883 μg/kg. Linkage map spanning 4060.6 cM developed consisted of 4 SSRs and 1780 SNPs. Seven linkage groups with an average distance of 2.3 cM between adjacent markers were identified. Four QTL regions with 36 putative QTL markers were identified (LOD scores ranged from 3.00 to 4.97). The identified QTL explained 6.3–16.9% of the phenotypic variation and were distributed across LG 2 and LG 5. The first report on construction of high-density linkage map through GBS for mapping QTLs for Fe uptake was published by Aldemir et al. (2017). They developed RILs from cross ILL 8006 × CDC Milestone. Fe concentration in RILs ranged from 37.2 to 175.7 mg/kg. A linkage map spanning 497.1 cM with 4177 SNP markers was constructed. Twenty-one QTL regions (exhibiting 5.9–14.0% of the phenotypic variation) were identified on six linkage groups (LG1, 2, 4, 5, 6, and 7). QTL mapping is routinely used for tagging and mapping of gene(s) of interest. However, QTL mapping suffers from the limitation of high cost, low resolution, and evolution of few alleles from two parents (Stich et al. 2006; Jannink and Walsh 2002).

Recently, association mapping has been used to identify QTLs in different crops. In legumes, limited reports are available on the use of AM for mapping gain Fe and Zn concentration like in chickpea (Diapari et al. 2014; Upadhyaya et al. 2016) and fieldpea (Cheng et al. 2015; Diapari et al. 2015; Kwon et al. 2012). In lentil, Singh et al. (2017) evaluated 96 germplasm lines for grain Fe and Zn concentration at three locations. The genetic variation in association mapping (AM) panel was characterized using a genetic distance-based and a general model-based clustering identifying six subpopulations. The study reported three SSRs (PBALC 13, PBALC 206, and GLLC 563) associated with grain Fe concentration (exhibiting 9–11% phenotypic variation) and four SSRs (PBALC 353, SSR 317-1, PLC 62, and PBALC 217) associated with grain Zn concentration (exhibiting 14–21% of phenotypic variation). Kumar et al. (2019) also evaluated 96 diverse lentil genotypes for three seasons for grain Fe and Zn concentration. The association mapping panel was genotyped using 80 polymorphic SSRs. Linkage disequilibrium analysis using Mixed Linear model revealed the association of two SSR markers GLLC 106 and GLLC 108 with grain Fe concentration (explaining 17% and 6% phenotypic variation) and three SSR markers PBALC 364, PBALC 92, and GLLC592 with grain Zn concentration explaining 6%, 8%, and 13% phenotypic variation, respectively. Khazaei et al. (2017) evaluated 138 cultivated lentil accessions (originating from 34 countries) for grain Fe and Zn concentrations for 2 years at two locations. The AM panel was genotyped with 1150 single-nucleotide polymorphism (SNP) markers. The marker–trait association (MTA) analysis identified two SNP markers linked to seed Fe concentration and one SNP linked to seed Zn concentration at $-\log10\ P \geq 4.36$.

7 Bioavailability and Limitations

Phytic acid is an antinutrient reported from legumes, cereals, oilseeds, and nuts. Phytic acid has an inhibitory effect on mineral bioavailability. Phytic acid binds with positively charged minerals, amino acids, proteins, and multivalent cations, resulting in the formation of complexes. Phytic acid chelates iron, zinc, manganese, calcium, copper, cobalt, and magnesium. DellaValle et al. (2013) reported positive correlation of grain Fe concentration with phytic acid concentration and negative correlation of Fe bioavailability with phytic acid concentration. Phytic acid is required for seed germination (Marshall et al. 2011). Thavarajah et al. (2009) examined 19 lentil genotypes grown at two locations for phytic acid concentration. They reported phytic acid concentration of 2.5–4.4 mg/g in lentil much lower than lpa mutants of soybean, common bean, wheat, and corn. It was further reported that decortications prior to cooking reduced total phytic acid by >50%.

Genetic biofortification with optimum phytic acid concentration has been proposed to enhance the micronutrient bioavailability. Efforts have been made in soybean to develop low phytic acid (lpa) mutant lines with reduced phytic acid with no adverse effect on germination (Vincent et al. 2015). Petry et al. (2013) demonstrated that consumption of lpa common bean iron absorption increased in

young women. Similarly, in lentil lpa mutants can help in increasing the bioavailability of minerals. The assessment of lentil genotypes for quality parameters is tedious and costly, requiring technical skill.

8 Future Thrust

Genetic variability in *Lens* genetic resources can be exploited to breed nutritionally improved lentil cultivars. Limited efforts have been made to evaluate global lentil genetic diversity for nutritional quality parameters. ICARDA, in collaboration with National Research Partners, can evaluate the core collections for micronutrients, protein content, phytic acid, and bioactive compounds. Nutritional traits in lentil are influenced by the environment. Some traits are expressed more in specific location; efforts should be concentrated on location-specific breeding of such traits. Limited efforts have been made to evaluate wild species for nutritional quality parameters. The useful accessions of wild species can be utilized for the development of prebreeding material. Genomic tools are routinely utilized in breeding programs. In lentil, good progress has been made in the development of genetic resources. The developed genomic resources can be exploited for biofortification of lentil. Based on multilocation phenotyping, association mapping for nutritional quality can be carried out using Genotyping by Sequencing. The QTLs mapped can be validated in biparental populations and utilized for marker-assisted selection. The marker-assisted breeding is cost effective and would accelerate the development of biofortified lentil.

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Biofortification of Mungbean

12

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Abstract

Mungbean is a highly nutritious and easily digestible grain legume known for a shorter crop duration, soil ameliorative properties, and wider adaptability. It fits well in numerous cropping systems as a sole crop as well as intercrop and records high per day productivity in comparison to several other pulses, cereals, and oilseeds, making it a viable option for economic sustainability of small and marginal farmers. Its grains are the primary economic produce that are consumed in several forms. Grown widely across different agro-climatic regions globally, it is an excellent and low-cost source of vegetable protein, iron, folate, potassium, and soluble fiber besides having low levels of fat, sodium, and glycemic index, making it one of the most preferred food legumes. Sprouting reduces antinutritional factors in mungbean seeds and enhances their overall nutrition quality, thereby increasing their economic value. Several other methods, viz., soaking, boiling, dehulling, and pressure cooking, are also reported to reduce antinutritional properties of mungbean seeds, thereby rendering them more usable

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and easily digestible. Systematic breeding efforts have been undertaken in mungbean in the past 5–7 decades, although most of these have remained confined to genetic improvement for yield and related traits, those too utilizing mainly the primary gene pool. Nonetheless, with more genetic and genomic resources becoming available, focus has gradually shifted toward the development of climate-smart and high-yielding mungbean genotypes with better nutritional qualities. Basic information has been generated for grain micronutrient concentration and their synthesis pathways, associated genes/QTL, linkage analysis, nutrient bioavailability, and containing antinutritional factors. This chapter focuses on all such developments and details biofortification of mungbean with reference to tackling protein energy malnutrition.

Keywords

Vigna radiata · Nutrition · Micronutrients · Sprouts · Molecular mapping · Nutrient bioavailability · Antinutrients

1 Introduction

The mungbean (*Vigna radiata* (L.) R. Wilczek var. *radiata*), popularly called green gram, is a nutritious and easily digestible legume crop that has gained tremendous economic importance in the recent years. It is an important constituent of the cereal-based farming systems of South and Southeast Asia while it is also grown in several other parts of the world including East and Central Asia, East Africa, and Australia. Currently, the global cultivation of mungbean spreads over 7.2 million ha with a productivity of about 750 kg/ha (Nair and Schreinemachers 2020). India is the largest producer, consumer, and importer of this short duration nutrition-rich crop, although Myanmar, China, Thailand, Indonesia, Kenya, Bangladesh, and Tanzania are also its major producers. India alone witnesses an area of 4.11 million ha with a production of 2.45 million tones and an average productivity of 596 kg/ha (Project Coordinator's Report, 2021). Nonetheless, a huge gap between the potential and realized yield is observed in mungbean with variable levels in almost all its production zones with the yield ranging between 0.4 and 2.0 tons/ha. A host of biotic and abiotic stresses significantly affect the production and productivity of mungbean (Pratap et al. 2020; Douglas et al. 2020; Nair et al. 2019, Singh et al. 2019), which mostly occur since mungbean is relegated to poorest of the soils with minimal inputs as it is mostly cultivated by small and marginal farmers. Incidentally, most of the breeding efforts in mungbean till date have remained focused on improvement of seed yield and stress resistance (Singh et al. 2017a; Pratap et al. 2019). Furthermore, the breeding efforts remained restricted to relatively few parental lines limiting the genetic diversity in cultivated mungbean (Pratap et al. 2019). Therefore, there is a need to broaden the narrow genetic base of cultivated mungbean, especially with respect to physical and nutritional quality enhancement of the seeds as well as the adaptive traits.

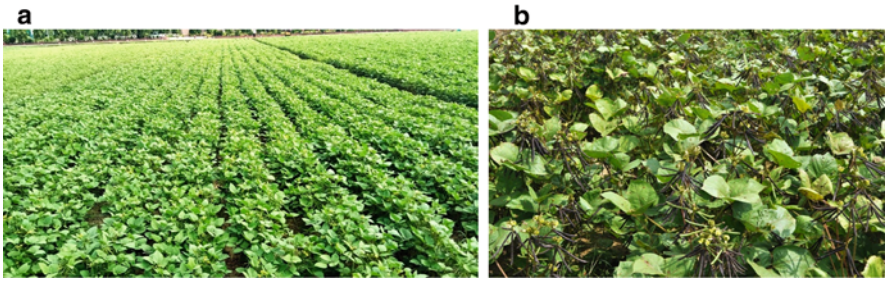

Fig. 12.1 An excellent crop of mungbean (**a**) at vegetative stage; (**b**) at maturity

Despite numerous production constraints, mungbean has a magical combination of several features, viz., shorter crop duration, low input requirements, wider adaptability, fairly good tolerance to heat and drought, and high nutritional content, which altogether make it an ideal crop for various cropping systems and patterns, especially for smallholder farmers (Pratap et al. 2020). The field view of mungbean at vegetative and reproductive stage is presented in Fig. 12.1. It offers the cultivators the dual advantage of a nutritious and priced farm produce as well as enhanced soil fertility after its harvest, which is mainly achieved by the symbiotic nitrogen-fixing soil Rhizobia. Like other pulses, mungbean also has numerous nutritional advantages over many other foods being an excellent source of vegetable protein, iron (Fe), folate, potassium (K), and soluble fiber (Singh and Pratap 2016). Pulses are gluten-free and, therefore, suitable for celiac people. Further, there have low-fat, low-sodium (Na), and low glycemic index and therefore better for lower glucose and insulin levels. Germination enhances the nutritional properties of mungbean significantly and makes them a premium breakfast food.

Of late, mungbean in being increasingly recognized as a potential pulse crop for cropping system diversification and sustainability and targeting possibilities as a functional food. Efforts are being reoriented toward the development of biofortified mungbean in several research institutes and also for sprouting purposes. This chapter discusses all nutrition-related aspects of mungbean including their nutrition content, genetic improvement and inheritance studies, use of genomic tools, new varieties, better crop management, and policy-related issues.

2 Nutritional Value

The nutritive value of mungbean lies in its highly nutritious and easily digestible grains as well as sprouts, which provide a significant amount of proteins (22–26%) and carbohydrates (62–65%) besides several other minerals. Mungbean seeds are reported to contain up to >31% protein (Itoh et al. 2006), although huge varietal difference has been observed as far as crude protein content is concerned (Das et al. 2015). The total protein content in mungbean protein isolates (MBPI) was reported to be 87.8% with total amino acid content of 800.2 mg/g (Kudre et al. 2013). The

seed storage proteins, namely globulin (60%) and albumin (25%), have been noticed in good enough quantity in mungbean (Ganesan and Xu 2018). It also contains remarkable quantity of essential amino acids like phenylalanine (1.443%), leucine (1.847%), isoleucine (1.008%), valine (1.237%), tryptophan (0.26%), arginine (1.672%), methionine (0.286%), lysine (1.664%), threonine (0.782%), and histidine (0.695%) (Mubarak 2005). However, a negative correlation between protein and methionine content in mungbeans has been reported (Yi-Shen et al. 2018). As mungbean is consumed in several forms, viz., husked, split-husked, and split dehusked, the protein content of the grain increases and the fiber content decreases with dehusking of the seeds. The seeds also contain several other essential elements including 1.4–1.85% fat, 3.5–6% fiber, 0.5–5.5% ash, and 62–67% carbohydrates on dry weight basis (Table 12.1). Lysine value of mungbean is high, and therefore, it is an excellent complement to rice in terms of balanced human nutrition. Dahiya et al. (2015) reported that the iron content in mungbean is also high and ranged from 5.9 to 7.6 mg per 100 g seed, thereby making it a highly nutritious crop for the lactating and pregnant women. Starch is the major constituent among the carbohydrates, and the total starch content was reported in the range of 40.6–48.9% of the seed in 20 mungbean cultivars studied in China (Shi et al. 2016). Due to his property, mungbean seeds are also utilized in food industry for noodle preparation (Nair and Schreinemachers 2020). The health-related properties of mungbean are presented in Table 12.2.

While pulses in general have remained grossly underestimated due to the so-called "antinutritive factors" relegating them to poor man's crops, mungbean has very less such factor in the form of unrelated chemical compounds that have varying effects on metabolic processes. However, most of these chemicals can be easily managed by various forms of preconsumption processing of the grains including soaking, sprouting, cooking, fermenting, and dehusking (Tajoddin et al. 2011). Pressure cooking usually digests the phytic acid, which interferes with mineral availability. Likewise, dehusking and germination may reduce the total tannin content. Sprouting is reported to reduce indigestible oligosaccharides, tannins, phytic acid, as well as the trypsin inhibitors in mungbean (Savage and Deo 1989) and, therefore, make the sprouts a much preferred and premium food. Wang et al. (2015) reported that germination led to a reduction of the phytic acid contents in mungbean by 76%. At the same time, the bioavailability value of zinc and iron increased by 3.0 and 2.4 times, respectively, as compared to the raw mungbean. The mungbean has also been reported to induce less flatulence (Dahiya et al. 2014) and therefore is well tolerated by children. Owing to its easy digestibility, high protein, and less flatulence-causing properties, mungbean has also been recommended as a supplement for preparing an infant's weaning food (Bazaz et al. 2016).

Kumar and Pandey (2020) recently reviewed the aspect of nutrient availability in soybean and mungbean and reported that the bioavailability of 5–15% for Fe and 18–34% for Zn, which need to be improved. They also gave attention for increasing the bioavailability of nutrients through breeding and agronomic practices coupled with emerging omics tools, which helps in preventing the malnutrition. Likewise, Majeed et al. (2020) reviewed different agronomic techniques for enhancing the

Table 12.1 Composition of the major nutrients in mungbean

Nutrition component	Range/100 g	Reference
Protein	14.6–33.0 g	Dahiya et al. (2015)
	22.0–25.0%	Kaur et al. (2020)
	23.0–29.0%	Augustine (1989)
	25.0%	Bhatty et al. (2000)
	20.97–31.32%	Anwar et al. (2007)
	22.90%	Agugo and Onimawo (2009)
	18.0–25.0%	Poehlman (1991)
	19.05–23.86%	Harper et al. (1996)
	26.6–30.0	Mubarak (2005)
Fat	1.45–1.85 g	Mubarak (2005)
Crude fiber	4.10–4.64 g	Mubarak (2005)
	3.8–6.15 g	Dahiya et al. (2013)
	5.03–12.63 mg	Harper et al. (1996)
	4.22 mg	Agugo and Onimawo (2009)
	5.9–7.6 mg	Dahiya et al. (2015)
	3%	Poehlman (1991)
	1–2%	Kaur et al. (2020)
	50%	Poehlman (1991)
	61.7–63.4	Mubarak (2005)
	55–60%	Kaur et al. (2020)
	53.3–67.1 g	Dahiya et al. (2013)
Phosphorus	340 mg	Kaur et al. (2020)
	367 mg	Poehlman (1991)
Calcium	247.67–277.3 mg	Harper et al. (1996)
	130 mg	Agugo and Onimawo (2009)
	132 mg	Poehlman (1991)
	118 mg	Kaur et al. (2020)
Amylose content	32%	Lang et al. (1999)
Ash	3.32–3.76 g	Mubarak (2005)
	0.17–5.87	Dahiya et al. (2013)
Energy	338–347 kcal	Dahiya et al. (2013)

bioavailability of Fe and Zn in mungbean. Ali et al. (2014a) also reported an increment in Fe concentration (46%) in mungbeans upon foliar application of Fe.

3 Uses of Mungbean

Mungbean is a highly preferred pulse crop that finds multifarious uses in local cuisine in several countries. It finds the most common use in the form of cooked/boiled dry grains with added spices known as *dal* (a kind of stew) in the entire South Asian regions (Pratap et al. 2021). *Dal* is usually prepared using whole grain as well

Table 12.2 Health-related properties of mungbean

Health-related property	Type of mungbean extract/ constituents (ose/reaction system)	Results of the study	Reference
Hypolipidemic property	Vitexin and isovitexin (25, 50, and 100 μM)	Lowered inflammatory cytokines	Inhae et al. (2015)
	Aqua extracts of raw, boiled, and sprouted mungbean (20 μL/220 μL)	Inhibited Alfa—glucosidase and Alfa amylase activity	Liyanage et al. (2018)
	Ethanolic extract of seed coat (5 mg/mL)	Inhibited α-glucosidase activity and decreased fat accumulation	Jang et al. (2014)
Anticancer property	Proteins isolated from mungbean aqueous extract (62.5, 125, 250, 500, and 1000 μg/mL)	Antiproliferation activities	Ketha and Gudipati (2018)
	Mungoin—a novel mungbean protease inhibitor (10, 50, 100, and 200 μM)	Antiproliferation activities	Yao et al. (2016)
	Phenolics (0.125, 0.25, 0.5, 1, 2, and 5 mg/mL)	Antiproliferation activities	Lee et al. (2013)
Antihypertensive property	Protein hydrolysates (5, 7.5, 10, 12.5, 15, 20, and 25 μg/mL)	ACE-I inhibitory activity	Xie et al. (2019)
	Vicilin protein (storage protein) hydrolysate (0.2–1.0 mg/mL)	ACE-I inhibitory activity	Gupta et al. (2018)
	Protein hydrolysate (100 μg/mL)	ACE-I inhibitory activity	Wang et al. (2006); Xu and Chang (2012)
Immunomodulation	Arabinogalactan (10, 50, 100, and 200 μg/mL)	Induced release of NO, TNF-α, IL-6, and IL-1β Increased phagocytic capability of macrophages	Ali et al. (2016)
	Water-extractable polysaccharides from mungbeans (50, 100, and 200 μg/mL)	Stimulate production of NO, TNF-α and IL-6	Luo et al. (2016)
	Saponins (50 and 100 μg/mL)	Inhibited Th cell proliferation	Yao et al. (2013)
	Aqueous extracts of untreated, germinated, and fermented mungbean (2.5 and 5 mg/mL)	Decreased NO level	Ali et al. (2014b)

(continued)

Table 12.2 (continued)

Health-related property	Type of mungbean extract/ constituents (ose/reaction system)	Results of the study	Reference
	Ethanolic extracts of whole mungbean, cotyledon, and hull (1 mL/5 mL)	Inhibited protease activity	Luo et al. (2016)
Anti-melanogenesis properties	Vitexin and isovitexin (10 and 15 μM)	Inhibited tyrosinase activity	Yao et al. (2013)
	Antityrosinase (20, 40, 60, 80, 120, 160, and 200 μg/mL)	Inhibited monophenolase and diphenolase activities	Chai et al. (2018)
	Ethanolic extract (15 mg/mL)	Inhibited tyrosinase activity	Kim et al. (2012)

as the split husked/dehusked grain. Consumers have particular preferences for grain size, seed coat luster (shiny or dull), and seed coat color (green or yellow) while they are using the whole grains for *dal*. For instance, in the entire Indian subcontinent, consumers prefer shiny green and small- to medium-sized grains, while shiny yellow and small grains (<3.0 g/100 seed) are preferred in Bangladesh, Sri Lanka, and some parts of India, especially northeastern states. On the contrary, consumers in Indonesia, Taiwan, Kenya, and Tanzania prefer dull green seeds. In Eastern India, consumers are reported to prefer mungbean with bright yellow seed coat and a particular pleasant aroma (Nair and Schreinemachers 2020), although most of the cultivars raised here are selections from landraces such as Sona mungbean. In India and parts of Southeast Asia, the fried and salted dehusked dry seeds of mungbean are a very popular snack. However, this kind of snack preparation requires specialty mungbean with medium-sized grains with thin seed coat and uniform texture. Mungbean floor is also used in the preparation of several sweet desserts while dehusked and overnight soaked mungbean grains are used for making porridge, candies, and an indigenous sweetmeat (*halua*). In Kenya and several African countries, mungbean is consumed as a thick bean stew. In many other countries, mungbean grains are consumed cooked with rice and also with sugar to make a sweet desert soup (e.g., in China), or grilled or roasted as a snack. Mungbean sprouts in one or the other form are consumed throughout the world, and owing to their enhanced nutrition properties fetch a premium segment of the vegetable protein market. Fresh mungbean spouts find an important place on the breakfast table as a cereal, side dish, soup mix, noodle garnish, as well as for stir frying and blanching purpose. As a result of a spurt in health consciousness among the elite class and an increasing trend of vegan concept, there has been a rising demand for mungbean sprouts, especially in high-income countries in Europe and North America. However, meeting the exacting quality standards for sprout mungbean and industrial production of sprouts still remains a challenge.

4 Importance in Alleviating Malnutrition

The mungbean is a miracle pulse crop with an excellent balance of several nutrients, including protein, minerals, vitamins, dietary fiber, as well as significant amounts of micronutrients. It is a comparatively low-cost source of good quality protein for those individuals who are either vegetarian or cannot afford animal proteins. Owing to its large-scale cultivation, wider adaptation, high nutrition, and easy digestibility, mungbean plays a significant role in alleviating protein energy malnutrition. Being a short duration crop, its per day productivity is quite high as compared to several other pulse and cereal crops and therefore its contribution in ensuring food and nutritional security is also proportionately high. It is a specially recommended food for the sick since mungbean protein is easily digestible as compared to protein in other legumes (Yi-Shen et al. 2018). Mungbean, when consumed in combination with the cereals, significantly increases the quality of protein and makes a balance of sulfur-containing amino acids present in cereals and lysine in mungbean (Boye et al. 2010) as Dahiya et al. (2015) suggested that a combination of mungbean protein with rice protein in 3:4 ratio provides the highest chemical amino acid score (72) and is excellent for human consumption. The saponin present in mungbean significantly reduces plasma cholesterol concentrations. Likewise, its fiber binds to the bile acids in small intestine and leads to reduced cholesterol, thereby reducing the risk of heart diseases.

Kumar and Pandey (2020) recently reviewed the aspect of nutrient availability in soybean and mungbean and reported the bioavailability of 5–15% for Fe and 18–34% for Zn, which need to be improved. They also suggested increasing the bioavailability of nutrients through breeding, and agronomic practices coupled with emerging omics tools, which helps in preventing the malnutrition. Likewise, Majeed et al. (2020) reviewed different agronomic techniques for enhancing the bioavailability of Fe and Zn in mungbean. Ali et al. (2014a) also reported an increment in Fe concentration (46%) in mungbean upon foliar application of Fe.

5 Genetic Resources for Grain Micronutrient Concentration

Harlan and de Wet (1971) postulated the gene pool concept that aimed at instituting a prebreeding program for directed crop improvement (Kumar et al. 2011). This has proved to be a colossal aid to breeders; since different species are organized into different gene pools, thus, for realizing a viable progeny, a breeder can determine which species is to be utilized in a hybridization program.

The origin of any species can chiefly be surmised through the presence of wild progenitors, in addition to the accessible archeological information of an area. Wild progenitors progressively diversify into cultivated species after evolutionary forces, viz., mutation, migration, hybridization, and genetic drift change their genomic constitution (Pratap and Kumar 2011). The center of origin of mungbean (*Vigna radiata* var. *radiata*) is believed to be the Indian subcontinent (deCandolle 1884; Vavilov 1926; Zukovskij 1962). Considering the wide range of genetic diversity of

Table 12.3 The gene pools of mungbean

Primary gene pool	Secondary gene pool	Tertiary gene pool	References
Vigna radiata var. *radiata*	*V. mungo* var. *mungo*	*V. angularis*	Chandel and Lester (1991), Smartt (1981, 1985)
V. radiata var. *sublobata*	*V. mungo* var. *silvestris*	*V dalzelliana*	
V. radiata var. *setulosa*	*V. aconitifolia*	*V. glabrescens*	
	V. trilobata	*V. grandis*	
		V. umbellata	
		V. vexillata	

Source: Kumar et al. (2011)

cultivated and wild species of mungbean found in India, it is contemplated to be the region of its first domestication. In cultivated and wasteland areas of India (Singh et al. 1974; Chandel et al. 1984; Lawn and Cottell 1988) and wetlands of subtropical northern and eastern Australia (Lawn and Cottell 1988), *V. radiata* var. *sublobata*, the progenitor species of mungbean, is found growing aplenty as a weed. Ample genetic variability is also found on roadsides, grasslands, and uninhabited areas of the Western *Ghats*. Table 12.3 details the secondary and tertiary gene pools of the genus *Vigna* after placing the cultivated species *V. radiata* in the primary gene pool. The individual species have been sorted according to their cross-compatibility together with cytogenetic, phylogenetic, and molecular evidence. The existence of useful genes is persistent in the secondary and tertiary gene pools (Tullu et al. 2006); but crossability barriers encountered during hybridization between the various *Vigna* species of the primary and secondary/tertiary gene pools necessitate the use of novel techniques (embryo rescue, polyploidization, reciprocal crossing, hormonal manipulations, use of bridge species) for realizing viable progenies (Pratap et al. 2018).

6 Classical Genetics and Traditional Breeding

The nutrient content of the edible portion of a crop available for consumption banks on several aspects such as the variety used, the location where the crop is grown, agronomic practices followed to raise the crop, and the storage conditions. For grain crops such as the mungbean, postharvest processes such as sprouting, dehulling, soaking, boiling, autoclaving, and microwave cooking also affect the composition of nutritional and antinutritional factors (Nair et al. 2013). Mungbean grains and sprouts produced from currently available varieties provide substantial amounts of protein (240 g/kg) and carbohydrate (630 g/kg) along with a range of micronutrients. Nonetheless, it is also true that till date very few systematic efforts have been undertaken to improve the nutritional quality of mungbean as most of the efforts were mainly directed toward improving its yield potential and biotic and abiotic

stress management. Now since numerous high-yielding, stress–resistant, and synchronous varieties have been developed with very high yield potential (Pratap et al. 2021), the efforts need to be focused toward improving its nutritional quality in terms of nutrients such as protein, carbohydrates, lipids, vitamins, minerals like iron and zinc, and other factors, including antinutrients like phytic acid.

6.1 Protein

The range of crude protein content found in mungbean grains is between 20.97% and 31.32% (Itoh et al. 2006). This high variation has been attributed to varietal differences (Yohe and Poehlman 1972; Thakare et al. 1988; Das et al. 2015), and different analytical methods employed (Dahiya et al. 2013) for crude protein estimation. Naik and Kole (2001) reported polymorphism in protein profiles in improved mungbean varieties and local land races from the state of Odisha, India. Total protein content in mungbean protein isolates (MBPI) was reported to be 87.8% by Kudre et al. (2013), with a total amino acid content of 800.2 mg/g. The constitution of essential amino acids was 43.5%, while sulfur-containing amino acids, viz., methionine and cysteine were about 1.6% of the total MBPI. A negative correlation has been noted between mungbean protein content and methionine content (Yi-Shen et al. 2018). Interspecific hybridization between mungbean and urdbean (blackgram), *Vigna mungo* L., has been attempted for transfer of high methionine content from blackgram into mungbean (Nair et al. 2013). Mature mungbean seeds have 8S globulin as the major storage protein; Torio et al. (2012) introduced methionine and cysteine residues into this storage protein through protein engineering, thus improving the amino acid score from 41 to 145%. The team also attempted to improve the protein quality by introducing free sulfhydryl groups and disulfide bonds to generate cysteine-modified mungbean 8Sa globulin protein. Chattopadhya et al. (2009) noted the existence of variation for trypsin inhibitor among mungbean varieties, which was proclaimed to be between 1324.26 TIU/g to 1502 TIU/g by Das et al. (2015). Yi-Shen et al. (2018) have reviewed the bioactivities for proteins and hydrolyzed peptides, including angiotensin-converting enzyme inhibitory activity, antifungal activity, and trypsin inhibitory activity, in mungbean.

6.2 Carbohydrates

Mungbean carbohydrates comprise starch components (available, resistant), fibers (lignin, cellulose), monosaccharides (maltose, glucose, xylose), and oligosaccharides (raffinose, stachyose, verbascose). Among these, starch is the major component, being utilized by the food industry for noodle preparation. Li et al. (2011) separated starch from 10 popular Chinese mungbean varieties and reported them to possess different physicochemical characteristics and diverse processing properties. Shi et al. (2016) reported that in 20 popular Chinese mungbean varieties the total starch content ranged from 40.6 to 48.9% of seed,

and that the resistant starch accounted for 16.1–22.3% of the total carbohydrates. Keenan et al. (2015) have stressed upon the potential of resistant starch to improve gut microbiota composition. Bean oligosaccharides are associated with flatulence after consumption; mungbeans cause less flatulence compared to other legumes (Goel and Verma 1981).

6.3 Lipids

Zia-Ul-Haq et al. (2008) reported that mungbean seeds have low (2.1–2.7%) oil content. The total tocopherol content of mungbean (12.5 mg/100 g) was reported to be higher compared to other legumes. Fatty acids palmitic (2.8–4 g), stearic (1.4–1.7 g), oleic (2.1–2.9 g), linoleic (3.4–4.6 g), linolenic (1.9–2.4 g), and arachidic (0.23–0.25 g) are found per kg of mungbean seed (Anwar et al. 2007). Adsule et al. (1986) and Abdel-Rahman et al. (2007) reported that linoleic acid was the most predominant and lauric acid was the least predominant of fatty acids found in mungbean.

6.4 Vitamins

Harina and Ramirez (1978) reported the presence of carotenoids as β-carotene and xanthophylls after evaluating 20 mungbean varieties differing in seed size and color. They elucidated that the carotenoid content in mungbean cotyledons (0.5–0.8 mg/100 mg) differs slightly between green and yellow varieties, while in seed coats (0.07–0.44 mg/100 mg) it varies tremendously between green and yellow varieties; further, the grain size has no correlation with the carotenoid content in mungbean. According to USDA (2010), on a per kg dry weight basis, vitamin A content is found to be higher in mungbean sprouts [100 lg retinol activity equivalent (RAE)] than grains (70 lg RAE). The vitamin C content ranges between 0 and 10 mg/100 g (dry weight basis) in mungbean (Prabhavat 1990); the vitamin C content in mungbean sprouts (1.38 g/kg, dry weight) is higher than in mungbean grains (0.05 g/kg, dry weight). The riboflavin content in mungbean is 0.29 mg/100 g (Nisha et al. 2005). Mungbean grains have a folate content of 0.0069 g compared with 0.0064 g for sprouts (per kg, dry weight basis) (USDA 2010). Using the stable isotope dilution assay, Rychlik et al. (2007) found 5-methyltetrahydrofolate as the predominant vitamin in mungbean and reported a folate content of 0.0028 g/kg dry mungbean seeds.

6.5 Iron

Dahiya et al. (2015) opined that the iron content in mungbean could range between 5.9 and 7.6 mg/100 g. Nair et al. (2015a, b) surmised that the iron content in Indian mungbean lines/varieties ranged between 3.5 and 8.7 mg/100 g, conceivably

providing 46–109% of RDA for males and 19–48% of RDA for females per 100 g mungbean consumed. The grain iron content may potentially be affected by agronomic factors, soil, and weather conditions (Thavarajah et al. 2009; Nair et al. 2015a, b). Mungbean varieties CN 9–5 and Harsha recorded almost double their iron content when grown in soils with high available iron (Nair et al. 2015a, b). A promising QTL (qFe-11-1) for iron was located on LG 11 map at the position of 113.7 cM by mapping in a recombinant inbred line population developed from a cross between ML446 (high iron content) and Sattya (low iron content) by Singh (2013).

6.6 Zinc

The zinc content in mungbean varieties varies from 2.1 to 6.2 mg/100 g (Taunk et al. 2011; Nair et al. 2015a, b). RAPD markers were employed to decipher zinc content diversity in local landraces of mungbean from Tamil Nadu, India (Karuppanapandian et al. 2006). Taunk et al. (2011) also employed RAPD markers to obtain polymorphism for zinc content in mungbean. Singh (2013) mapped a RIL population developed from a cross between ML446 (high zinc content) and Sattya (low zinc content) and found four promising QTLs (qZn-11-4, qZn-11-5 on LG 11 and qZn-4-1, qZn-4-2 on LG 4) at a map distance of 196.2 cM, 296.3 cM, 13.7 cM, and 87.9 cM, respectively.

6.7 Other Minerals

While investigating the distribution of different minerals in mungbean plant, Singh et al. (1968) established the presence of 812 mg/100 g (dry weight) of calcium (about 30–50%) in the seed coat, 23 mg/100 g (dry weight) of iron in the embryo and 17 mg/100 g (dry weight) of iron in the seed coat, and 756 mg/100 g (dry weight) of phosphorus in the embryo and 341 mg/100 g (dry weight) of phosphorus in the cotyledons. Nair et al. (2015a, b) examined popularly cultivated mungbean lines/varieties of South Asia for variability in mineral content and proclaimed the ranges for various minerals, viz., calcium (1190–1580 mg), magnesium (970–1700 mg), zinc (21–62 mg), copper (7.5–11.9 mg), manganese (9.8–19.6 mg), selenium (0.21–0.91 mg), potassium (8670–14,100 mg), and phosphorus (2760–5170 mg) per kg dry weight. The authors also opined that the variation in the concentration of minerals could be due to the effect of environment in which they were cultivated as well as due to the method of determination of these minerals, viz., inductively couple plasma-emission spectrometry (ICP–EMS) (Nair et al. 2015a, b), atomic absorption spectrometry (Barakoti and Bains 2007), and EDTA titration method (Kadwe et al. 1974).

6.8 Phytic Acid

Dahiya et al. (2013) expounded phytic acid, tannins, hemagglutinins, polyphenols, trypsin inhibitor, and proteinase inhibitor as the antinutritional components in mungbean. Phytic acid is the main seed storage molecule for phosphorus. Low phytic acid content is desirable since high phytic acid can reduce the bioavailability of iron, zinc, and other mineral micronutrients. Sompong et al. (2010a), while investigating 250 mungbean accessions for variations in phytic acid content, noted the range to be between 1.8 and 5.8 g/kg dry grain. They also established that high phytic acid content was controlled by dominant alleles at two independent loci showing duplicated recessive epistasis. Along with this, high broad-sense heritability (80%) registered for phytic acid content implied that breeding for low phytate content was feasible. Sompong et al. (2010b) pinpointed a few mungbean QTLs with low or moderate effect on phytic acid content, but some of these QTLs overlapped with QTLs for seed size, flowering, and maturity, thereby restricting their use through MAS. Nair et al. (2015a, b) have also recorded low phytic acid content (2.6–3.8 g/kg) in mungbean varieties/lines. However, these variations recorded in phytic acid content could also be due to the method of analysis employed, viz., estimation of the myo-inositol hexaphosphate content by anion exchange HPLC separation (Lestienne et al. 2005) or phytic acid extracted using 0.5 M HNO_3 and determined colorimetrically (Grewal and Jood 2006). Since phytic acid is also essential for seed development and germination, breeding for reduced phytic acid content should not be detrimental to seed germination (Bohn et al. 2008).

6.9 Other Compounds

Mungbean exhibits hemagglutination activity (Mubarak 2005) through sugar-binding proteins that bind with red blood cells and agglutinate them, causing lesions and improper microvillus development of the epithelial cells, leading to abnormal absorption of nutrients. Trypsin inhibitor activity [56–98 trypsin inhibitor units (TIU) mg/protein] and tannin content (3.1–4 g/kg grain) (Philip and Prema 1998) as well as saponins (5.7 g/kg dry weight; Fenwick and Oakenfull 1983) are shown. Additionally, bruchid (*Callosobruchus* spp.) infestation during storage leads to increased trypsin inhibitor activity (25%), saponin level (16%), and phytic acid content (46%) (Modgil and Mehta 1994). Development of bruchid-resistant mungbean varieties (Nair et al. 2015a, b) can mitigate the risk of losses in nutritional quality during storage. Cao et al. (2011) explored the antioxidant properties of flavonoids in mungbean and established the presence of vitexin and isovitexin (more than 96%) in the seed coat. Mungbean sprouts were reported to contain higher levels of total phenolic and flavonoid extracts [0.167–0.192 g ferulic acid equivalent (FAE) per kg dry weight] compared to dry seeds (0.098–0.101 g FAE per kg dry weight) (Kim et al. 2012), thereby both having the potential for therapeutic use (Yao et al. 2008, 2011a, b). Attar et al. (2017) identified aroma volatiles and deciphered the 2-acetyl-1-pyrroline biosynthetic pathway in aromatic mungbeans.

7 Brief on Diversity Analysis

Comparing the nutrient levels in landraces and improved varieties of mungbean, Ebert et al. (2017) established that at full maturity the older mungbean accessions were superior in protein, calcium, iron, zinc, carotenoid, and vitamin C content than the modern improved mungbean lines. The genetic enhancement of mungbean is therefore attainable in terms of protein quality, starch content and quality, content of minerals like iron and zinc, and reduction in antinutritional compounds like phytic acid. Indirectly, progress can also be made by tackling other traits such as resistance to bruchids, which otherwise cause huge losses during storage and also lead to a reduction in the nutritional quality of the stored grains. Seed size has been reported to have a nonsignificant correlation with micronutrient content; therefore, breeding for large-seeded mungbean varieties will in no way impact the nutrient composition of the seed, thereby eliminating the danger of losing the nutritive value of the grain by developing small- or large-seeded varieties (Nair et al. 2015a, b). Nonetheless, large-seeded varieties with 100-seed weight >4.0 are less preferred by the consumers in its major consumption areas (Stakeholders meet, ICAR-IIPR, Kanpur, Feb. 09, 2019). Nutritive value of sprouts over grains has been emphasized several times (Ebert et al. 2017), and varieties with better grain nutrient content would definitely have increased nutritive value as sprouts. Mungbean has an added advantage compared to other legumes that both protein and carbohydrates are easily digestible and create less flatulence. In mungbean, lower phytic acid concentration (2.6–3.8 g/kg) compared to other pulses may lead to increased bioavailability of micronutrients (Nair et al. 2015b). Mungbean has been used as Fe-rich whole food source for baby food due to its nutritional quality and palatable taste. WorldVeg identified mungbean lines that have the capacity for improved uptake of iron from the soil (Nair et al. 2015a, b), and these lines have been utilized in the breeding program for improving the iron content in the commercial varieties.

8 QTLs and Genomics-Aided Breeding for Biofortification

Mungbean and other *Vigna* species including its progenitor and nonprogenitors are considered highly nutritious legumes (Rehman et al. 2019). It contains easily digestible vegetarian proteins and other important micronutrients (Akaerue and Onwuka 2010; Kollárová et al. 2010). Nonetheless, it is a less studied crop for biofortification-related traits as compared to other legumes (Dwivedi et al. 2012). Generally, mungbeans contain higher concentrations of micronutrients than cereals, oilseeds, or root crops, but still require improvement for bioavailability (Blair 2013). Biofortification is the process of genetic improvement for increasing nutritional values and reducing antinutritional factors in the edible seeds (Pfeiffer and McClafferty 2007; Dwivedi et al. 2012).

Quantitative trait loci (QTL) analysis through biparental, advanced backcross, NAM, and MAGIC populations or genome-wide association mapping in natural populations using molecular different markers system and sequencing techniques

provide ways to identify the potential QTLs and genes underlying biofortification-related traits (Blair 2013). The QTL studies for mineral nutrients in other legumes have earlier been studied in mapping populations of *Lotus japonicus* (Klein and Grusak 2009) and *Medicago truncatula* (Sankaran et al. 2009). After the decoding of the whole-genome sequence (Kang et al. 2014), the genetic studies on mungbean have been sparse. Some of the researchers reported the low polymorphism of SSRs in mungbean (Tangphatsornruang et al. 2009). A number of researchers (Humphry et al. 2002; Chen et al. 2015; Liu et al. 2017) developed the applied SSR markers for deciphering genetic diversity in mungbean. Asides, some of the researchers (Kitsanachandee et al. 2013; Gupta et al. 2013; Singh et al. 2020) used the transferable SSRs from cowpea and adzukibean for tagging QTLs. Singh et al. (2017c) constructed a linkage map spanning 2919.7 cM distance in mungbean RILs panel and 17 QTLs (2 for iron and 15 for zinc content) were identified on four linkage groups (LG4, LG6, LG7, and LG11). They identified the genomic regions as qZn-4-3 and qFe-4-1 on chromosome 4 between PVBR82-BM210 markers; qZn-11-2 and qFe-11-1 on chromosome11 between BM141-BM184 markers, which were co-located on the same chromosomal regions for Zn or Fe, which probably were closely linked to each other, or were the same pleiotropic QTLs. Van et al. (2013) discovered over 300,000 SNPs in mungbean, among them only 43 and 20 SNPs have been validated as competitive allele specific polymorphism (KASP) markers in the two studies conducted to date (Van et al. 2013; Islam and Blair 2018). These markers can be further utilized in mungbean breeding. Genotyping by Sequencing (GBS) based on faster development of next-generation sequencing (NGS) technology is one of the most important alternatives to single marker assays for SNPs (Poland and Rife 2012), which can be used for polymorphism discovery (Elshire et al. 2011) for a wide range of crops (He et al. 2014). To date, limited studies on GBS have been undertaken in mungbean for genetic mapping and diversity assessment (Schafleitner et al. 2016; Noble et al. 2018). Wu et al. (2020) performed SNP-based association mapping for micronutrients in 95 mungbean genotypes representing 13 countries. They identified about 6486 SNPs and 43 marker–trait associations (MTAs) for calcium, iron, potassium, manganese, phosphorous, sulfur, or zinc concentrations in mungbean seeds. These MTAs were scattered across 35 genomic regions explaining 22% of the variations on an average. Of these, 11 regions were associated with seed macronutrients, 12 with micronutrients, and 12 with other elements. Three genes on chromosome 1, namely *Vradi01g00820*, *Vradi01g00830*, and *Vradi01g00840*, and one on chromosome 5, that is, *Vradi05g16350*, were associated with K and P concentrations. Two genes *Vradi07g26320* and *Vradi07g26340*, on chromosome 7 were near SNPs associated with P concentration. Likewise, three genes (*Vradi07g14180* on chromosome 7, and *Vradi08g22740* and *Vradi08g17100* on chromosome 8) were identified as associated with K. Genes related to Fe accumulation such as *Vradi06g09900* (metal iron binding), *Vradi06g10020* (metal translocation), *Vradi06g10060* (mineral uptake), *Vradi06g10120* (membrane transport), and *Vradi06g10210* (ATP binding) were located on chromosome 6. Four genes, viz., *Vradi01g05570*, *Vradi07g05950*, *Vradi07g06200*, and *Vradi06g02380*, were found associated with Zn content, which

were involved in gene regulation, metal translocation, metal iron binding, and membrane signal transduction pathways. Four SNPs were associated with Mn concentration, in which only one gene *Vradi01g11650* was found in their vicinity, which were reportedly involved in carbohydrate metabolic process unrelated to Mn accumulation.

8.1 Brief Account of Molecular Mapping for Grain Micronutrient Concentration

Micronutrient malnutrition is a growing concern in the developing world. It causes diverse health and social problems, like mental retardation, impairments of the immune system, etc. (Ghandilyan et al. 2006). Iron deficiency affects 3.7 billion people while zinc deficiency affects 49% of the human population (Welch 2002; Brown et al. 2001). Zinc is required as a cofactor in over 300 enzymes. It helps in the formation of DNA-binding domain (Palmer and Guerinot 2009). In recent years, the zinc (Zn) deficiency problem appears to be the most serious micronutrient deficiency together with vitamin A deficiency. Iron is a very important micronutrient to produce red blood cells (RBCs) and maintain hemoglobin. Fe deficiency would result in lower hemoglobin (Grotz and Guerinot 2006). As compared to other micronutrients, the deficiency of iron and zinc is the most prevalent disorder throughout the world (Jeong and Guerinot 2009). In general, nutritional deficiencies are prevalent in developing countries where people do not have diverse diet of vegetables, fruits, and meat or fish, so grain crops are by necessity the major source of essential nutrients for humans (Dwivedi et al. 2012). Biofortification is the best option to enhance the Fe and Zn content with little recurring costs (Chandel et al. 2011). Therefore, to enhance the iron and zinc content in mungbean seed is the best way to alleviate the deficiency of iron and zinc (Singh et al. 2013a). For developing a variety with high concentration of iron and zinc, it is a foremost thing to identify germplasm with high concentration of both (iron and zinc) micronutrient and to understand their genetic mechanism (Singh et al. 2013b).

Quantitative trait loci (QTL) analyses through mapping populations or genome-wide association studies (GWAS) using molecular markers provide valuable ways of identifying the genes underlying nutritional traits (Blair 2013). The quantitative trait loci (QTL) give a powerful genetic approach to characterize the candidate gene and allele mining (Vert et al. 2002). However, only a few reports are available for the identification of QTLs in iron and zinc micronutrient content. Therefore, it is of great importance to study the molecular mechanisms of iron and zinc accumulation in mungbean seed. The use of QTLs related to micronutrient content can reduce the time and cost to develop new cultivars with improved nutritional value. Sufficient variability has been reported in mungbean for seed Fe and Zn content (Beebe et al. 2000). However, there are very few studies on identifying genes for iron and zinc regulation in mungbean. The use of QTLs/molecular markers linked to micronutrients can speed up the development of biofortified new cultivars.

Aneja et al. (2012) studied molecular diversity among 21 mungbean genotypes using 29 sequence-related amplified polymorphism (SRAP) markers. These genotypes had varied Fe (29.95–100.97 mg kg^{-1}) and Zn (20.13–35.70 mg kg^{-1}) content. They reported that the SRAP analysis could not group the genotypes based on the micronutrient content. Taunk et al. (2012) studied genetic diversity among the 16 mungbean for iron and zinc content using RAPD markers. They reported that iron and zinc concentrations varied from 46.31 to 106.15 and 23.31 to 40.46 mg kg^{-1} dry grain, respectively. They also reported that high Fe and Zn content genotypes were not clustered together and were also able to identify low and high Fe and Zn content genotypes.

Singh et al. (2013c) studied the genetic diversity for iron and zinc content using RILs of two crosses with AFLP markers. They reported wide variation for iron (1.6–9.3 mg/100 g) and zinc (1.5–3.9 mg/100 g) content in both RIL populations. However, they were unable to report any QTLs for the iron and zinc content. Sompong et al. (2012) identified QTLs for phytic acid P (PAP), total P (TP), and inorganic P (IP) in mungbean seeds and seedlings from the F_2 population of a cross between low PAP cultivated mungbean (V1725BG) and high PAP wild mungbean (AusTRCF321925). Seven QTLs were detected for P compounds in seed; two for PAP, four for IP, and one for TP. Six QTLs were identified for P compounds in seedling; three for PAP, two for TP, and one for IP. Only one QTL colocalized between P compounds in seed and seedling, suggesting that low PAP seed and low PAP seedling must be selected for at different QTLs. Seed PAP and TP were positively correlated with days to flowering and maturity, indicating the importance of plant phenology to seed P content.

Singh et al. (2017c) have identified QTLs for mungbean seed Fe and Zn content in recombinant inbred line (RIL) population between ML776 and Sattya. A large genetic variation and transgressive segregation was observed for Fe and Zn content. Linkage map was developed, which spanned 2919.7 cM distance. A total of 17 QTLs (2 for iron and 15 for zinc content) were mapped on four linkage groups, viz., LG 4, LG 6, LG 7, and LG 11. The genomic regions qZn-4-3 and qFe-4-1 on chromosome 4 between PVBR82-BM210 markers; qZn-11-2 and qFe-11-1 on chromosome 11 between BM141-BM184 markers were co-located on the same chromosomal regions for Zn or Fe content. These were probably closely linked to each other or same pleiotropic QTLs. The SSR markers associated with QTLs for both high iron and zinc content would be useful in marker-assisted breeding for biofortification in mungbean.

Wu et al. (2020) have identified 6486 high-quality single-nucleotide polymorphisms (SNPs) from the genotyping by sequencing (GBS) dataset and found 43 marker × trait associations (MTAs) with calcium, iron, potassium, manganese, phosphorous, sulfur, or zinc concentrations in mungbean grain. The MTAs were scattered across 35 genomic regions explaining on average 22% of the variation for each nutrient. Other SNPs identified will serve as important resources to enable marker-assisted selection (MAS) for nutritional improvement in mungbean and to analyze cultivars of mungbean. In this study, 9 out of 12 SNPs associated with Fe and Zn were located on chromosomes Vr06 and Vr07. Even though different

methods were used to locate genomic regions/genes for Fe and Zn concentration in mungbean, the overlapping regions indicated common genomic regions that are responsible for Fe and Zn accumulation in mungbean. The Yellow Stripe Like (YSL) proteins are members of the oligopeptide transporter family and acting as a transporter of iron and metal-nicotianamine chelates responsible of iron loading of the seeds (Jean et al. 2005). Wu et al. (2020) also found a gene similar to YLS7 on chromosome Vr07, but this one was associated with Zn accumulation.

8.2 Association Mapping Studies

Association mapping or linkage disequilibrium (LD) studies emerge as a powerful approach for mapping economically important traits using unstructured diverse germplasm. This approach is based on principal of linkage disequilibrium (LD), which refers to the nonrandom assortment of nonhomologous chromosomes during meiosis (Lewontin and Kojima 1960). It is a commonly used substitute approach to biparental mapping for the identification of genomic region controlling for natural variation in phenotypic/biochemical or other traits of interest. It is a sturdy genetic mapping tool for many crops including mungbean and provides high-resolution, broad allele coverage, and cost-effective gene tagging for the evaluation of plant germplasm resources.

9 Extent of Linkage Disequilibrium

Mungbean has largely remained a crop of subsistence agriculture with limited genetic information available, particularly on micronutrient concentration of grains and its improvement has relied on traditional plant breeding methodologies for most of its cultivated history (Fernandez et al. 1988; Humphry et al. 2002; Pratap et al. 2019). Based on available genome sequence database, it is having relatively small genome size 543 Mb for understanding genetic diversity and evolutionary pathway (Kang et al. 2014). Previous genetic diversity studies of cultivated and wild mungbean germplasm (Pratap et al. 2015a, b), using both morphological and molecular markers (Mohan et al. 1997), have highlighted low levels of genetic diversity in cultivated mungbean compared to the broader diversity found in wild mungbean (Saravanakumar et al. 2004; Sangiri et al. 2007; Pratap et al. 2012). To date, knowledge of the genetic basis for many important agronomic traits, such as seed coat color, grain size, flowering time, and disease resistance, relied on linkage and quantitative trait locus (QTL) analysis using segregating populations derived from either intra-specific or inter-specific crosses. Traditionally, linkage mapping is the fundamental tool to identify genetic loci underlying traits of interest. A limited number of genetic linkage maps have been developed in mungbean (Lambrides et al. 2000; Humphry et al. 2002; Isemura et al. 2012). Despite great efforts, a comprehensive and saturated genetic linkage map of all 11 chromosomes has not been generated (Kim et al. 2015). Instead, high-density maps developed from

whole-genome sequences (Kang et al. 2014) enable further advancement in alternative approaches to trait dissection, such as association mapping, also known as linkage disequilibrium (LD) mapping (Gupta et al. 2005; Abdurakhmonov and Abdukarimov 2008; Noble et al. 2018). LD mapping takes advantage of historical recombination events in a diverse set of lines to identify the genetic basis of traits at a higher resolution than traditional genetic linkage mapping. The resolution of association mapping relies upon the extent of LD. The degree of LD has yet to be accurately determined in mungbean. In the recent study, a mungbean diversity panel consisting of 466 cultivated and 16 wild accessions was characterized and in total over 22,000 polymorphic genome-wide SNPs were identified. Observed average polymorphism information content values of 0.174 versus 0.305 in wild mungbean, LD decay in _100 kb in cultivated lines and a distance higher than the linkage decay of _60 kb were estimated in wild mungbean (Noble et al. 2018). Likewise, in another study, a total of 5041 SNPs were used for genotyping of 293 mungbean accessions that passed strict filtering for genetic diversity, linkage disequilibrium, population structure, and GWAS analysis, and the results reveled that polymorphisms were distributed among all chromosomes, but with variable density (Sokolkova et al. 2020). They reported that linkage disequilibrium decayed in approximately 105 kb (Sokolkova et al. 2020). Although the LD method has been used increasingly over the last decade for the study of complex genetic traits in many plant species including legumes, a limited number of studies have reported marker–micronutrient (Fe, Zn, and Se) associations in pulse crops such as mungbean and urdbean. Therefore, it can be inferred that identification of molecular markers for selecting genes associated with increased micronutrient accumulation is just initiated in mungbean.

9.1 Genome-Wide LD Studies

Food legumes usually contain higher concentrations of micronutrients than the cereals or root crops. However, these still need improvement for their total concentration as well as bioavailability. The recent release of a reference genome for mungbean (Kang et al. 2014) provides new opportunities for mungbean genomic research (Kim et al. 2015; Pratap et al. 2014a, b). Therefore, quantitative trait loci (QTL) analyses through mapping populations or genome-wide association studies (GWAS) using molecular markers and high-throughput sequencing techniques provide valuable ways of identifying the genes underlying nutritional traits (Blair 2013). Genome-wide association studies (GWAS) have recently emerged as a powerful approach for finding genetic variation for micronutrients in germplasm. This approach has been used in a range of food legumes, from soybean (Hwang et al. 2014), common bean (Blair et al. 2016), lentil (Ates et al. 2018), and the model legume *Medicago truncatula* (Kang et al. 2019) to several other legumes. To date, GWAS have been undertaken in mungbean for genetic mapping and diversity study (Schafleitner et al. 2016; Noble et al. 2018), but not for association mapping for specific micronutrients of grains. Although mungbean potentially contributes to the

alleviation of iron, zinc, and protein deficiency in human populations of several Asian countries (Singh et al. 2017b), no significant genetic research efforts for improving mineral nutrients have been undertaken in this crop. Most recently, Wu et al. (2020) conducted genome-wide association study (GWAS) for nutrient concentration based on a seven mineral analysis using inductively coupled plasma (ICP) spectroscopy in 95 cultivated mungbean genotypes chosen from the USDA core collection representing accessions from 13 countries. They identified a total of 6486 high-quality single-nucleotide polymorphisms (SNPs) from the GBS dataset and found 43 marker–trait associations (MTAs) with calcium, iron, potassium, manganese, phosphorous, sulfur, or zinc concentrations in mungbean grains produced in either of two consecutive years' field experiments. The MTAs were scattered across 35 genomic regions explaining on average 22% of the variation for each seed nutrient, and this study will serve as important resources to enable marker-assisted selection (MAS) for nutritional improvement in mungbean.

9.2 Application of Association Studies for Germplasm Enhancement

Although conventional breeding approaches have been successful to address the issue of low productivity in mungbean, this is not happening at the desired success rate. Therefore, it is very essential to intensify the legume genetic enhancement programs using advanced breeding approaches wherein the potential of genomics needs to be exploited for accelerated development of improved cultivars possessing high-yield, genetic resilience against stresses, and enhanced nutritional quality. Although biparental mapping has been successful in identifying many significant quantitative trait loci (QTL) mapped to wide intervals in the mungbean genome, our knowledge of genes controlling certain traits is still limited. The GWAS methodology became well established in plant genetics during a decade of great effort. It is considering much more recombination events by using an association panel of individuals, each of those potentially characterized by a unique recombination history. Several commercial microarrays were designed for large-scale genotyping and analysis of GWAS panels, with many accompanied tool kits developed. Thus, GWAS approaches have been widely used in genetic research to identify the genes involved in many legumes including mungbean. With the rapid development of sequencing technologies and computational methods, GWAS are now becoming a powerful tool for detecting natural variation underlying complex traits like micronutrients of grain in mungbean.

10 Genetic Engineering for Enhancement of Micronutrients

Even though mungbean is largely consumed as an iron-rich whole food for babies, the bioavailability of the micronutrients is of concern. With the bioavailability of Fe and Zn ranging in between 5–5% and 18–34% of total food intake, a lot more is

needed to compensate for the low availability (Kumar and Pandey 2020). Even though mung possesses low phytic acid, which is known to affect bioavailability of micronutrients, and the pulse has easy digestible protein, there is still scope for improvement in micronutrient content. Again, presence of hemagglutinins, fiber, and heavy metals also inhibits bioavailability of micronutrients (Thavarajah et al. 2014). While there are many studies reporting biochemical studies for understanding the micronutrient composition of mungbean and variations among varieties, there are upcoming reports on genomics-aided efforts for the development of micronutrient-rich mungbean. Reports on gene(s)/QTLs specific for Fe and Zn in mung are awaited.

Genetic transformation systems have been well developed in mungbean and several genetic transgenics have been developed, but the majority of them are proof of concepts (Jaiwal et al. 2001; Mahalakshmi et al. 2006; Islam and Islam 2010; Bhajan et al. 2019) and remaining target abiotic stress tolerance traits (Baloda et al. 2017; Mekala et al. 2016; Yadav et al. 2012). With the availability of a deep-sequenced reference genome of Asian mungbean, freely available data on SNPs, tagged germplasm for specific traits, and easy regulatory policies, GEd mungbean using SNP–trait associations will mark the future.

11 Nutrient Bioavailability

Mungbean protein is rich in essential amino acids, such as total aromatic amino acids, leucine, isoleucine, and valine. On the contrary, mungbean protein is slightly deficient in threonine, total sulfur amino acids, lysine, and tryptophan (Mubarak 2005). The starch present in mungbean is easier to digest as compared to that of many other food legumes such as chickpea, pigeon pea, and lentil (Sandhu and Lim 2008). Because of its high protein content and hypoallergic properties, mungbean has also been advocated as a supplement for preparing infants' weaning food (Bazaz et al. 2016; Ali et al. 2016). It has been observed that the protein digestibility of the rice mungbean combination diet is 84.4% of that observed for the rice–meat combination diet in infants, which can almost meet human needs for protein (Hussain et al. 1983).

The true digestibility of mungbean was reported to be 73% (Tsou and Hsu 1978; Mubarak 2005). Likewise, the protein efficiency ratio of mungbean is 4.29, which is quite high, whereas the essential amino acid index is 67.8. The rat-feeding experiments showed that a combination of 75% protein from rice and 25% protein from mungbean gives a protein efficiency ratio equivalent to 75% of casein protein (Tsou and Hsu 1978).

11.1 Phytic Acid

Phytic acid has been reported to provide resistance to the grains against the bruchid beetle (Srinivasan et al. 2007) and therefore is advantageous from a storage

perspective. However, it has a negative impact on iron and zinc bioavailability. Mungbean has been observed to be lower in phytic acid (72% of the total phosphorus content) as compared to pigeon pea and soybean (*Glycine max*) (Chitra et al. 1995). To date, only naturally occurring germplasms that possesses low PA have been identified in mungbean (Sompong et al. 2010a). Due to its palatable taste and nutritional quality, mungbean has been used as an iron-rich whole food source for baby food (Imtiaz et al. 2011). Nair et al. (2015a, b) observed that the mungbean lines commonly grown in South Asia have significant variations for Fe (35–87 mg/kg), Ca (1190–1580 mg/kg), Mg (970–1700 mg/kg), Zn (21–62 mg/kg), Cu (7.5–11.9 mg/kg), Mn (9.8–19.6 mg/kg), Se (0.21–0.91 mg/kg), K (8670–14,100 mg/kg), and P (2760–5170 mg/kg). The Fe concentration of lines CN 9-5 and Harsha doubled when grown in soil with increased availability of Fe. The low PA concentration (2.6–3.8 g/kg) and the presence of phenolic compounds such as ferulic acid (1540–3400 mg/g) in mungbean may lead to increased bioavailability of micronutrients.

11.2 Hemagglutinins

Hemagglutinins are the sugar-binding proteins that bind with red blood cells and agglutinate them. They bind with specific receptors at epithelial cells of the intestine, causing lesions and improper microvillus development, leading to abnormal absorption of nutrients. El-Adawy et al. (2003) and Mubarak (2005) investigated hemagglutinin activity in mungbean and did not show much variation.

11.3 Polyphenols

Mungbean contains a considerable amount of polyphenols that affects the nutrient digestibility and bioavailability adversely. Although phenolics are present in the cotyledons as well as seed coats of mungbean, but most of them are concentrated in the seed coats. The polyphenol content also varies depending upon many factors, viz., variety, seed coat color, and climatic and agronomic conditions. Mungbean contains several major phenolic components in variable quantities such as phenolic acids (1.81–5.97 mg rutin equivalent/g), flavonoids (1.49–1.78 mg catechin equivalent/g), and tannins (1.00–5.75 mg/g) (Lee et al. 2011; Shi et al. 2016; Singh et al. 2017a, b). Salunkhe et al. (1982) reported that polyphenols are present in higher amounts in colored and darker legume varieties than in the light-colored varieties, which suggests that improvement through breeding is possible. Simultaneously, the seed coat color of the mungbean grains can be used as a marker for the selection of varieties with lower amounts of polyphenols. This also indicates that the mungbean products made of yellow or light-colored mungbean varieties could have higher protein digestibility and mineral bioavailability since polyphenols have been observed to reduce the protein digestibility and mineral bioavailability. Muhammed et al. (2010) suggested that the seed coat polyphenols can help the seed against

pathogens and improve seed viability. Therefore, the yellow mungbean varieties may be cultivated for better yields. At food processing level, polyphenols can be reduced subsequently by using various processing methods.

11.4 Starch

Consumption of low glycemic index food is useful in the reduction of diabetes mellitus and obesity (Westman et al. 2008; Noakes et al. 2015). The low glycemic index diet is beneficial in normalizing the diet-insulin responses by improving adipocyte insulin-mediated glucose uptake in vitro (Ludwig 2002). Interestingly, mungbean starch has an added advantage of having a low glycemic index. The mungbean starch contains a higher level of amylose than that of other pulses (Hoover et al. 2010). Kabir et al. (1998) compared the effects of the chronic consumption of mungbean starch (32% amylose) and cornstarch (0.5% amylose) on a glucose metabolism in normal and diabetic rats and observed that the rats fed with the high amylose content of mungbean starch showed a lower glycemic index in comparison to the rats fed with waxy cornstarch with low content amylose. Therefore, due to low glycemic levels of the mungbean, its use in developing new products can help to prevent the risk of diabetes.

12 Enhancement of Promoters and Reduction of Antinutrients

12.1 Phytic Acid

A small amount of antinutritional factors present in mungbean may hinder the biological value of available nutrients. For example, the phytic acid can bind with iron, zinc, calcium, and magnesium, leading to the formation of insoluble complexes. These insoluble complexes can limit the absorption of minerals and their utilization in the small intestine (Weinberger et al. 2002). However, various processing methods, viz., fermentation, germination, dehulling, soaking, and cooking, can reduce the antinutritional factors (Islam and Ali 2002; Barakoti and Bains 2007; Hemalatha et al. 2007). After germination, the phytic acid contents declined in mungbean by 76%, and bioavailability values of zinc and iron increased were 3.0 and 2.4 times higher than that of raw mungbeans, respectively (Nair et al. 2012). Therefore, the antinutritional properties can be efficiently managed by various processing methods and do not hinder the use of the mungbean.

12.2 Trypsin Inhibitors

Trypsin inhibitors affect protein digestion adversely by inhibiting proteolytic enzymes. Trypsin inhibitor in mungbean does not inhibit chymotrypsin as well as

vicilin peptidohydrolase (Chrispeels and Baumgartner 1978). Interestingly, trypsin inhibitor activity of mungbean is much lower as compared to that of soyabean, kidney bean, and chickpea (Guillamón et al. 2008). Trypsin inhibitors are low molecular-weight proteins and thus likely to leach during soaking. Therefore, germination and soaking lower the trypsin inhibitor activity. Trypsin inhibitor activity is also reduced by heat treatments (Chandrashekar et al. 1989).

13 Germination or Sprouting

Germination is a complex metabolic process in which the lipids, carbohydrates, and storage proteins present in the seed are broken down to provide energy and amino acids. While germination is the simplest natural process and improves digestibility and availability of certain nutrients, the extent of positive impact of germination depends on the type of the legume and also on the conditions and duration of the germination process (Savelkoul et al. 1992). It has been observed that sprouting reduces the antinutritional factors and improves overall nutritional quality (Malleshi and Klopfenstein 1996) of food legumes. As a result of controlled germination, the minerals like calcium, zinc, and iron are released from bound form. Phytic acid is reduced, so the availability of minerals is increased during germination (El-Adawy 2002).

Sprout production is a simple germination process and does not have any season limitation in mungbean. The process is completed within a short period and also with very limited facilities including only seeds, sprouting containers, and water as inputs. Although several kinds of legumes may be consumed as sprouts, mungbean, chickpea, and cowpea are the preferred legumes for sprouting purpose. Yang and Tsou (1998) reported that the iron content of mungbean sprouts varied between 0.072 and 0.095 g kg^{-1}. Available iron content in sprouts increased owing to the increased ascorbic acid content and reduced phytic acid content during sprouting. Initial ascorbic acid levels increase on average from 0.129 to 0.228 g kg^{-1} after sprouting in mungbean varieties "NM 98" and "Ramzan."

It has been observed that controlled germination reduces the levels of reducing sugars and starches significantly by 36.1% and 8.78%, respectively (Mubarak 2005). They however noted that until 60 h of incubation levels of the monosaccharides fructose and glucose increased dramatically in the germinating material. However, significant reductions in the levels of both sugars were observed after 60–75 h. Likewise, the concentration of the disaccharide sucrose also increases within the first 24 h, but rapidly declines after the initial germination phase (El-Adawy et al. 2003). The decline of sucrose in the latter stages of sprouting may be due to the lack of raffinose, resulting in the hydrolysis of sucrose for the energy supply (Mubarak 2005). Further, raffinose and stachyose are also observed to be completely eliminated during germination.

Kataria et al. (1989) observed a reduction in phytic acid content during sprouting. After 96 h of sprouting period, Shah et al. (2011) recorded a reduction in the phytic acid level from 1.88 to 0.33%. A similar result was reported by Yang and Tsou

(1998). The reduced phytic acid might be attributed to leaching of the antinutrients into soaking water under the influence of concentration gradient, which governs the rate of diffusion (Kakati et al. 2010). The loss of phytic acid during germination may be caused by hydrolytic activity of the enzyme phytase. Similar losses of phytic acid during soaking and germination have been reported by Grewal and Jood (2006). Kim et al. (2012) found that the total phenolics, total flavonoids, and antioxidant activity measured with DPPH radical scavenging were higher in mungbean sprouts than in seeds. Tannin content was reduced by 66.7% through sprouting and by 45.5% through boiling; phytic acid content was also reduced by 25.9% with boiling. The amount of hemagglutinin can be reduced by germination (El-Adawy et al. 2003).

Guo et al. (2012) reported that after germination the phenolic acids and flavonoids, including vitexin and isovitexin, increased significantly, up to 4.5 and 6.8 times in the sprouted mungbean, than that of raw mungbean seeds. Likewise, Ebert et al. (2015) reported that the vitamin C content of mungbean sprouts is 1.7-fold higher than that of soybean sprouts and 2.5-fold higher than that of amaranth sprouts. Ebert et al. (2017) observed a 7.9-fold increase of total ascorbic acid content in mungbean sprouts compared to mungbean grain. Ascorbic acid (vitamin C) is the most potent enhancer of iron absorption. Vitamin C content is low in mungbean grains (0.05 g kg^{-1} dry weight) but high in sprouts (1.38 g kg^{-1} dry weight). The scope for genetic improvement of vitamin C content in mungbean grains is very limited compared with the postharvest processing through sprouting.

13.1 Soaking

Soaking has been reported to reduce the phytic acid content of mungbean and the complementary food items based on mungbean and/or rice flour, thereby leading to enhanced bioavailability of iron and zinc in the body (Perlas and Gibson 2002). Soaking mungbean prior to cooking is a common practice in Indian households, and this unknowingly helps in improving its nutritional value and reducing antinutritional factors. In Andhra Pradesh, India, *pesarattu* dishes commonly consumed in local diets are made by soaking mungbean seeds and then grinding the seeds into a paste. Khalil (2006) reported that fermentation also helped to reduce the phytic acid level (30–38%) as well as trypsin inhibitor activity (19–63%) in mungbean. Khattab and Arntfield (2009) reported that soaking caused a 42.82–48.91% reduction in phytic acid content. This could be due to the fact that phytic acid in dried legumes exists wholly as a water-soluble salt presumably as potassium phytate (Crean and Haisman 1963).

13.2 Dehulling

Dehulling during the process of milling and *dal* making is found to be very useful in reducing the polyphenol content. Muhammed et al. (2010) reported that dehulling

significantly reduced the polyphenol content of mungbean by 14–52%. It is also a common practice to consume mungbean in India and many Southeast Asian countries after removing the seed coat, particularly for *dal* purpose and also for sweetmeats, which is often followed by soaking, offering the dual advantages. Nonetheless, a significant portion of mungbean is also consumed without removing the seed coat. In Kerala, India, a dish is prepared by boiling whole mungbean grains with spices and serving the cooked mungbean with rice. Tajoddin et al. (2010) observed that total polyphenols ranged from 2.8 to 3.56 g kg^{-1} in whole mungbean seeds, from 7.02 to 12.96 g kg^{-1} in the seed coat, and from 1.72 to 2.86 g kg^{-1} in the cotyledons.

13.3 Pressure Cooking

According to Kakati et al. (2010), the antinutritional factors in mungbean and blackgram were significantly reduced after various processing treatments. They suggested that pressure cooking led to 35.2% and germination to 33.4% decrease of phytic acid content over the seed, which remained unprocessed. An increase in the period of pressure cooking is effective in reducing antinutritional factors (Sinha et al. 2005). Crean and Haisman (1963) had also reported that during the process of cooking phytic acid combines with the calcium and magnesium of the seeds, leading to the formation of insoluble calcium and magnesium phytates. Therefore, the reduced phytate value observed during different processing treatments might be attributed to the heat effect and changed permeability of seed coat. Tannin content present in raw seeds of greengram showed a sequential decline with pressure cooking followed by soaking and germination. Germination of greengram seeds reduced tannin content to a greater extent compared to other treatments. Among the various processing treatments, pressure cooking was found to be most effective in the retention of the nutrients. Thompson et al. (1983) suggested that a high-temperature treatment during processing reduced hemagglutinins in red kidney beans. Likewise, polyphenols are also reduced by roasting and leaching during soaking (Barroga et al. 1985).

14 Social, Political, and Regulatory Issues

Legumes are the most important protein source for the vast majority of vegetarian population in Asia, especially the thickly populated Indian subcontinent. Mungbean offers a great promise in achieving the nutritional security is such a scenario and therefore finds an important place in the area and production expansion plan of pulses in major pulse-growing countries such as India (Vision 2030 and vision 2050, ICAR-IIPR, Kanpur). It has been envisioned to bring an additional area of 3–-4 million ha under pulses including about 1 million ha in mungbean alone by promoting mungbean in rice and wheat fallows, intercropping with sugarcane and vegetables and intensifying different cropping systems. Clearly, if a sustainable

development of mungbean production has to be achieved, a three-pronged strategy needs to be adopted, which mainly includes (1) vertical expansion of the crop by improving the yield potential of mungbean cultivars, (2) horizontal expansion by extending its cultivation in new areas, and (3) intensifying well-established cropping systems with integration of shorter duration cultivars. Timely availability of quality seeds of improved varieties is a serious issue in most of the mungbean-growing countries. Varietal mismatch and late arrival of quality seeds are the two most common problems in all mungbean-growing areas, which require an immediate attention. To address this issue, the Department of Agriculture and Cooperation, GOI, has established 150 seed hubs in the country to ensure availability of 1000 quintals of quality seeds of pulses through each seed hub every year. Mungbean finds an important place in many seed hubs, and these seeds have been producing seeds of mungbean varieties, which have been developed in only the last 10 years. Likewise, 12 "enhancing breeder seed production centers" have been established to ensure breeders seed production of mungbean and other pulses.

Fewer varieties for each pulse producing agro-climatic zones will ensure availability of pulses. Larger areas under single or fewer varieties will help in adopting suitable crop management and mechanization. Identification of varieties with uniform size and shape minimizes adjustments in machine parameters, thus minimizing the loss in the form of breakage. Storage losses accounting to 15–30% loss in all stored grains and mungbean are no exception. The current storage protocols adopted for storage of mungbean are similar to those in major cereal. There is a strong need to develop specific storage protocol for mungbean. Jute bags prone to internal and external infestations are still being used for pulse storage, whereas for export and import PP woven bags are used. Adoption of PP woven or HDPE bags at storage level will minimize the chances of external infestation. Initial infestation can be curbed by fumigation of fresh arrival.

Buffer stock should be created for longer periods, at least for 5 years and only 1/5 part need to be replenished with fresh crop. This will minimize transportation cost and losses. Further, the buffer stock should be converted into *dal* prior to release; otherwise, millers will dictate the market. Nonetheless, for all of these targets, the cultivation of mungbean has to be made less cost intensive and profitable to farmers. Unfortunately, short duration crops like mungbean come associated with drudgery, especially when most of the crop is still harvested by hand picking.

15 Future Perspectives

Mungbean evidently contains a magical balance of essential nutrients and bioactive compounds, and with its rich profile of polysaccharides, polyphenols, and polypeptides it has obvious nutritional and pharmacological properties and also qualifies as a functional food crop. Owing to its health and nutritional benefits as well as soil and environment ameliorative properties, mungbean promises to be a preferred candidate crop for food, nutrition, and environmental security and sustainability. Realizing its importance as a nutrition-rich crop, studying various

bioactive compounds in detail was advocated in as early as in the 1960s (Savage and Deo 1989), which has been reiterated in numerous recent studies also and fortunately a renewed interest is there in studying such compounds that could add value to the mungbean products. Processing prior to consumption leads to a significant increase in mungbean properties while reducing its antinutritional properties. Likewise, postharvest processing and value addition enhance its commercial value tremendously. However, this aspect has not been given the part of attention it actually deserved. At the same time, a restraint is required on overemphasis on the antinutritional properties of the pulses, in general, and mungbean, in particular, since these can be easily and effectively managed through various simple and cost-effective hose-hold processing methods. Several in vitro and in vivo studies have indicated beneficial health effects of mungbean; nonetheless, the mechanisms involved in disease prevention and the metabolic processes leading them to become a functional food are essential to unravel.

Ample variability exists in different mungbean cultivars and germplasm accessions for almost all nutrition-related traits, suggesting that improvement for most of these is possible through simple breeding methods. The sprouts segment represents the high-value segment of the market, although the grains need to meet exacting quality attributes. This is an area that requires high-quality standards and, therefore, higher investments. Pesticide residue, nonuniform grain size, and hypocotyl pigmentation are some of the issues that need strict monitoring and quality standard compliance. The first step in this direction would be identification of suitable varieties and their cultivation on a large area over the same season so as to obtain uniform produce. At the same time, the policies need to be framed by the governments, which ensure timely availability of quality seeds of varieties specifically suited to the purpose.

For milling of pulses, such varieties must be identified, which record higher dal recovery in milling, indicating lower gum content in between husk and cotyledons. While efforts must be made to consume pulses as whole to prevent milling losses, pulses in form of *dal* are better protected from bruchid infestation as it does not provide hiding space to insect larvae and can be stored for longer duration. About 30% whole grain is lost in form of milling by-product, which is rich in proteins and antioxidants. Presently, it goes for low-value cattle feed. Powder component of the milling by-product can be separated and can be utilized as source for pulse proteins, whereas husk rich fraction can be used as nutraceuticals.

Postharvest storage has a great role to play in maintaining the nutritional and physical qualities of all pulses. Bruchids (*Callosobruchus* species) cause huge losses to the stored grains in terms of both physical and nutritional quality. These are reported to enhance trypsin inhibitor activity, saponins, and phytic acid in the stored grains (Modgil and Mehta 1994). Therefore, there is an urgent need to identify bruchid-resistant donors, molecular markers associated with resistance, and initiate host–plant resistance breeding immediately. There is a need of international collaborative efforts toward exploitation of biological variation for various nutritional parameters and deploy strong and reliable analytical methods to determine nutritional compounds in mungbean.

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Biofortification of Chickpea

13

Shailesh Tripathi, Rajesh Kumar Singh, Swarup K. Parida, S. K. Chaturvedi, and P. M. Gaur

Abstract

Chickpea with potential health and nutritional benefits has been and is being consumed by human beings since ancient times. Seeds of chickpea serve as a staple source of protein in human diets, especially for those with vegetarian food habit. Enhancement of the nutritional composition of chickpea and other food legumes has the potential to combat micronutrient malnutrition besides having beneficial effects on human health. Fe and Zn content in seed appears to be a complex trait under control of number of genes/QTLs and the concentration of these nutrients is highly influenced by edaphic and environmental factors. An integrated genomics approach involving Quantitative trait loci (QTL) mapping, association analysis, and differential gene expression profiling is currently the most efficient strategy for genetic dissection of complex traits like yield and nutritional quality. Evaluation of chickpea genetic resources has revealed significant variation for protein content as well as micronutrients. The present review highlights the efforts being made towards screening of germplasm, mapping of QTLs for seed protein and micronutrient content, and future thrust areas for enhancing the nutritional quality of chickpea.

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Keywords

Cicer arietinum · Protein · Micronutrient · Genetic variation · Biofortification

1 Introduction

Chickpea, a member of the family *Fabaceae*, is one of the major food legumes grown in more than 50 countries across the globe. Chickpea (*Cicer arietinum* L.) is a self-pollinated, annual diploid, and highly nutritious food legume with a genome size of ~740 Mbp (Varshney et al. 2013; Jain et al. 2013). It is the second most important food legume crop after common beans (*Phaseolus vulgaris* L.). The crop is widely cultivated across continents in Asia, Africa, Australia, Europe, North America, and South America. There are two morphologically distinct types of chickpea, desi and kabuli. Desi types are pink flowered with pigmented stem and thick colored seed coat while kabuli are white flowered without anthocyanin pigmentation on stem and have white or beige colored seeds which are typically ram's head shape. The desi types, predominantly grown in Asia and Africa, have a major share in chickpea cultivation and are grown in 80–85% of the total chickpea area.

Vavilov suggested fertile crescent (Southwest Asia) and the Mediterranean as possible center of origin with South Asia and Ethiopia as secondary centers. Varshney et al. (2019), based on the population difference index (F index) suggested a migration route from the Mediterranean/Fertile Crescent to South Asia (India) further to East Africa and Central Asia. The data supports the movement of large-seeded, cream-colored chickpeas from East Africa to India via Central Asia about two centuries ago, apparently through Afghanistan, and hence its name kabuli chickpea (in Hindi).

Chickpea with potential health and nutritional benefits has been and is being consumed by human beings since ancient times. Seeds of chickpea serve as a staple source of protein in human diets, especially for those with vegetarian food habit. Chickpea consumption is known to have beneficial effects on human health by lowering the risk of some important human diseases such as cardiovascular disease, type 2 diabetes, digestive disorders, and some types of cancers (Jukanti et al. 2012). In biofortification, the aim is to increase the nutrient density of food crops through conventional plant breeding, and/or improved agronomic practices and/or biotechnological approaches without compromising on any characteristic preferred by end-users (Nestel et al. 2006). The identification of micronutrient-rich staple food can be a sustainable and long-term solution to address the problem of micronutrient malnutrition or so-called hidden hunger. Enhancement of the nutritional composition of chickpea and other food legumes has the potential to combat micronutrient malnutrition besides having beneficial effects on human health (Rehman et al. 2018). According to an estimate by 2050, around 1.4 billion women and children would be iron deficient and 175 million zinc deficient (Smith and Myers 2018). The year 2016 was declared as the "International year of pulses" with the aim of highlighting the

benefits of pulses and legumes in human diets and their contribution to sustainable food production, food and nutritional security, and reducing poverty (FAO 2016).

1.1 Economic Importance of Chickpea

At present, chickpea is grown in over 50 countries spread across Asia, Africa, Australia, Europe, and the Americas. During the past decade, there has been impressive growth in the chickpea area and production. Globally, during 2018 the crop was grown in an area of about 18 m ha with a production of 14.4 mt (FAOSTAT 2019). India is the major chickpea growing country with a share of more than 65% in production. The chickpea production in the country rose steadily during the last two decades from a mere 3.86 mt during 2000–2001 to a record high of 11.2 mt during 2017–2018 (Agricultural Statistics at a Glance 2019). The chickpea revolution in India is evident in the central and southern states of Madhya Pradesh, Maharashtra, Rajasthan, Andhra Pradesh, Karnataka, and Gujarat which have shown a remarkable increase in area and production. Despite being the major producer of chickpea, India remains a major importer of desi chickpea because of increased domestic demand for consumption. However, in the past decade, India has started exporting kabuli chickpea mainly to Pakistan, Algeria, Turkey, Sri Lanka, UAE, etc. It is projected that the total pulse production in the country has to reach around 39 million tonnes by 2050 in order to attain self-sufficiency and major portion (16–17.5 mt) of this has to come from chickpea itself (Dixit et al. 2019).

1.2 Nutritional Value of Chickpea

Ensuring food and nutritional security to the growing human population will be a major challenge before the agricultural scientists especially in the era of climate change and resource scarcity. Adequate intake of nutritious food containing all essential micronutrients has become a prerequisite for healthy living. More than 3 billion people worldwide, including children in developing countries, are suffering from micronutrient malnutrition (Welch and Graham 2004; Thavarajah and Thavarajah 2012). Assuming similar micronutrient bioavailability and retention after cooking or processing and storage, persons will consume and absorb more micronutrients from eating biofortified crops than from same amount on nonfortified crops (La Frano et al. 2014; De Moura et al. 2015).

Chickpea is a good source of carbohydrates, protein, dietary fiber, vitamins, and minerals (Fig. 13.1). It has the potential to ameliorate protein and micronutrient deficiencies. Chickpea seeds are significantly rich in all essential amino acids (except sulfur-containing amino acids). Chickpea seed protein is considered the best among all legumes and generally varies from 17% to 22% (of total dry seed mass) across the core and mini-core set, landraces, and cultivated desi and kabuli germplasm accessions (Upadhyaya et al. 2002, 2006; Jadhav et al. 2015). The in vitro protein digestibility of chickpea was found to be higher compared to pigeon pea, mungbean,

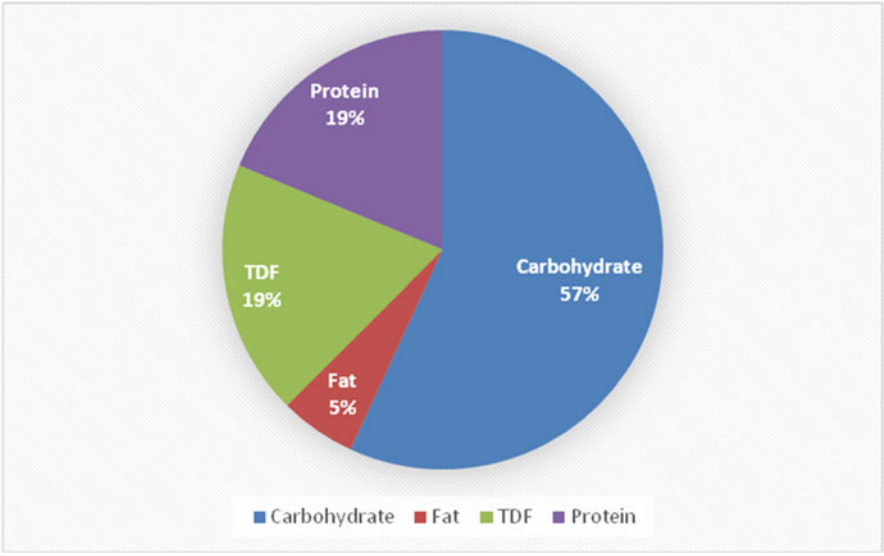

Fig. 13.1 Nutritional composition of chickpea seed (Jukanti et al. 2012)

urdbean, and soybean (Chitra et al. 1995). The consumption of chickpea with cereals provides a balance of all essential amino acids in the diet by complementing each other for limiting amino acids (lysine in cereals and sulfur-containing amino acids in chickpea).

Adequate intake of nutritious food enriched with essential micronutrients is a prerequisite for humans to meet their metabolic needs and maintain good health. Iron and zinc deficiency are the most prevalent nutritional problem globally (Platel and Srinivasan 2016). Iron is involved in many important metabolic processes like respiration, synthesis of DNA, and cell proliferation. Zinc deficiency leads to several health problems like growth retardation, impaired brain development, poor wound healing, tissue hypoxia, hypogonadism, diarrhea, infertility, and increased risk of infections (Crook 2011). Substantial variability is present in the chickpea germplasm for seed iron and zinc concentrations (Diapari et al. 2014; Grewal et al. 2020). Chickpea has an average of 3.0–14.3 mg of Fe, 2.2–20.0 mg of Zn, and 334–446 kcal/100 g edible portion (Petterson et al. 1997; Wood and Grusak 2007; Ray et al. 2014). Many approaches such as mineral supplementation, dietary diversification, and food fortification are being followed to overcome the problem of micronutrient deficiency or hidden hunger. These efforts have had met with little success due to lack of social and cultural awareness and inappropriate socioeconomic infrastructure (White and Broadley 2005).

Currently, the chickpea breeding programs around the world aim at increasing yield and resilience to biotic and abiotic stresses. This has led to an increase in global chickpea production but not much attention has been paid towards improving its nutritional quality. Use of genomics-assisted breeding to identify and introgress gene

(s)/QTLs (Quantitative Trait Loci) controlling Fe and Zn content can be deployed in chickpea for its biofortification.

2 Evaluation of Genetic Resources for Grain Micronutrients

Micronutrient malnutrition is not only due to food insufficiency but also because of poor nutritional quality of the available food. Reduced bioavailability of nutrients like iron and zinc in plant-based foods due to the presence of anti-nutritional factors, like phytate, phenolics, and fibers, is another major factor responsible for micronutrient deficiency (Raes et al. 2014). Phytic acid chelates micronutrients and reduces their bioavailability as monogastric animals, including human beings, do not have enzyme phytase in their digestive tract (Gupta et al. 2015). Increase in carbon dioxide levels in the atmosphere primarily due to climate change, also lead to an increase in phytic acid content in the food grains, which in turn affects the micronutrient content in food crops (Myers et al. 2014). Fe and Zn content in seed appears to be a complex trait under control of number of genes/QTLs and the concentration of these nutrients is highly influenced by edaphic and environmental factors.

Chickpea is considered as an excellent whole food which is a good source of dietary proteins, carbohydrates, micronutrients, and vitamins (Jukanti et al. 2012). Raw chickpea seed on average contains 5.0 mg/100 g of iron, 4.1 mg/100 g of zinc, 138 mg/100 g of Mg, and 160 mg/100 g of calcium (Fig. 13.2). About 100 g of chickpea seed can meet daily dietary requirements of iron (1.05 mg/day in males and 1.46 mg/day in females) and zinc (4.2 mg/day in males and 3.0 mg/day in females)

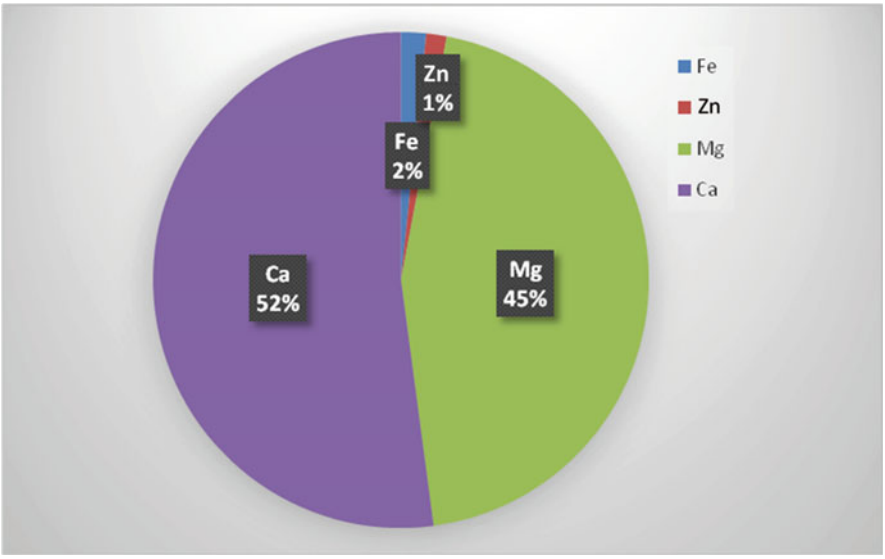

Fig. 13.2 Mineral composition of chickpea seed (Jukanti et al. 2012)

Table 13.1. Mineral composition of chickpea seeds

Minerals (mg/100 g)	Rao and Deostahle (1981)	Ibanez et al. (1998)		Wang and Daun (2004)		Diapari et al. (2014)	Grewal et al. (2020)
		Desi	Kabuli	Desi	Kabuli		
Copper	1.18	1.25	1.20	0.5–1.4	0.7–1.4	–	–
Iron	4.60	4.51	4.46	4.6–7.0	4.3–7.6	3.62–8.64	0.5–8.54
Zinc	6.11	3.57	3.50	2.8–5.1	3.6–5.6	1.86–6.22	1.1–5.91
Manganese	1.21	1.72	1.65	2.8–4.1	2.3–4.8	–	–
Calcium	220	210	154	115–226.5	80.5–144.3	–	–

(FAO 2002). Grewal et al. (2020) in their study on a set of 402 genotypes consisting of released varieties, breeding lines, landraces, core and composite collection indicated the presence of genotypic diversity for seed iron and zinc content in chickpea. Zinc content in chickpea germplasm ranged from 1.10 to 5.91 mg/100 g while iron content was between 0.50 and 8.54 mg/100 g (Table 13.1.). The released varieties contained relatively higher amounts of Zn and Fe compared to germplasm from core and composite collection and landraces.

Genetic variation for Fe (36.2–86.4 mg/kg) and Zn (18.6–62.2 mg/kg) was reported in 94 diverse chickpea accessions (Diapari et al. 2014). Several accessions with high Fe and Zn were identified. Three kabuli accessions (CDC Verano, ILC 2555, and FLIP 85-1C) accumulated highest concentrations of Fe and Zn with an average of 60.1, 59.2, and 58.8 ppm, respectively, of Fe and 48.3, 44.6, and 42.6 ppm, respectively, of Zn.

2.1 Mapping for Grain Iron and Zinc Content

An integrated genomics approach involving Quantitative trait loci (QTL) mapping, association analysis, and differential gene expression profiling is currently the most efficient strategy for genetic dissection of complex traits like yield and nutritional quality, in diverse crop plants including chickpea (Kujur et al. 2015). An intraspecific $F_{2:3}$ population derived from the cross between MNK-1 and Annigeri was phenotyped for seed Fe and Zn and genotyped using genotype by sequencing approach in order to map QTLs controlling seed Fe and Zn content in chickpea (Syed et al. 2020). Genetic linkage map was constructed with 839 single nucleotide polymorphisms (SNPs) spanning 108,804 cM with average marker density of 1.30 cM. A total of 11 QTLs located on linkage groups 3, 4, and 5 for seed Fe concentration which explained 7.2–13.4% phenotypic variation were identified. While for seed Zn concentration 8 QTLs spanning linkage groups 4, 5 and 8 and explaining 5.7–13.7% phenotypic variation were identified. Two QTLs for seed Fe

concentration and one for Zn concentration were co-localized along with QTLs for drought tolerance-related traits in the "QTL hotspot" region on CaLG04.

2.2 Evaluation of Genetic Resources for Protein Content

Trait association analysis, selective genotyping, and differential expression profiling have been used to dissect the complex seed protein content (SPC) in chickpea. Genotyping-by-sequencing (GBS) information from a population of 336 desi and kabuli accessions was used to carry out genome-wide association study (GWAS) (Upadhyaya et al. 2016). Seven genomic loci associated with SPC (explaining 41% of total phenotypic variance) were identified. Five of the SPC-associated genes (encoding ATP-dependent RNA helicase DEAD box, cystathionine-beta synthase, CMP and dCMPdeaminases, G10 and zinc finger protein) were validated in the parental accessions and homozygous individuals of a recombinant inbred line (RIL) mapping population developed from the cross ICC12299 × ICC4958 by selective genotyping. The presence of identical low and high SPC-associated alleles derived from these five gene-derived SNP loci in the parents (ICC12299 and ICC4958) and homozygous contrasting mapping individuals was observed. The integrated genomic approach delineated diverse naturally occurring novel functional SNP allelic variants in six potential candidate genes controlling SPC. They reported a strong association between SPC trait and a non-synonymous SNP allele carrying zinc finger transcription factor gene.

Inheritance of protein content in chickpea was studied in a cross between desi chickpea line ICC5912 (protein content 29.2%) and ICC17109 (protein content 20.5%). The F2 population showed continuous distribution for seed protein content indicating that it is a quantitative trait controlled by multiple genes (Gaur et al. 2016). Genome-wide association studies on 187 chickpea accessions identified 4 QTLs for seed protein content and the variation explained by these marker trait associations ranged from 2.4% to 5.1% (Jadhav et al. 2015).

2.3 Breeding for High Carotenoid Concentration

Chickpea seeds contain carotenoids such as beta-carotene, cryptoxanthin, lutein, and zeaxanthin in amounts greater than the engineered beta-carotene containing "Golden rice" and can be a good potential source of dietary carotenoids. Carotenoids are reported to increase the natural killer cell activity (Santos et al. 1998). Vitamin A is a derivative of beta-carotene, is involved in several important developmental processes in humans like bone growth, cell division and differentiation, and above all vision. Millions of children develop xerophthalmia (damage to cornea) and about 250,000–500,000 million children become blind due to vitamin A deficiency. Thus, breeding for high carotenoid concentration in the seeds has nutritional and socioeconomic importance.

3 Conclusion and Future Thrust

Chickpea is and will continue to remain an important pulse crop in India. The chickpea improvement programs across the globe need to be dynamic have to focus on the traits preferred by farmers, industries, and consumers. Ample of scope exists for further improving the nutritional quality of chickpea. Protein content of the present-day chickpea cultivars is usually in the range of 18–22% whereas large variation (upto 32%) exists in the chickpea germplasm which can be tapped either through conventional or molecular breeding to develop protein-rich ($\geq$25%) varieties. Also, the variability for Fe and Zn content in chickpea can be exploited for enhancing the Fe and Zn content in chickpea. Genomics-assisted breeding can be integrated into the chickpea improvement programs for increasing the efficiency and precision of quantitative traits like nutritional quality. The availability of draft genome sequence and use of next-generation sequencing can successfully provide an insight into naturally occurring genetic variability in chickpea germplasm. With the whole genome information coupled with phenotypic study, it is possible to identify rare variants that may contribute to a key phenotype such as abiotic stress tolerance or nutritional quality traits. The comprehensive chickpea hapmap with 4.97 million SNPs (Varshney et al. 2019) is a valuable resource for undertaking high-resolution genome-wide association studies (GWAS). The identification of molecular signatures or candidate gene(s) governing protein, Fe, and Zn content in chickpea seed will be useful for translation genomics to breed cultivars with superior nutritional quality.

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Prospects of Biofortification in Groundnut Using Modern Breeding Approaches

14

Sai Rekha Kadirimangalam, Rachana Bagudam, Murali T. Variath, and Janila Pasupuleti

Abstract

Biofortification is the roadmap to deliver micronutrients to the malnourished population with limited access to a healthy nutritious diet. Among the nutritious food crops, groundnut is being consumed to an extent of 4 million metric tons as part of the human diet. Groundnut or peanut is a rich source of protein (~25%), fat (~50%), dietary fiber, vitamins, and minerals; thus, peanuts and peanut-based food products are used globally to treat protein, energy, and micronutrient malnutrition. Though groundnut has a good proportion of nutrients, there is a scope to further enrich it with micronutrients like Fe and Zn, increase the oil for use in energy bars, and oleic acid content of the fat for improved human health, especially in developing and underdeveloped countries. Groundnut breeding approaches, along with advanced genomic techniques, enable the development of nutrient-rich groundnut that can save millions of malnourished people around the world. Genetic variability is available for oleic acid, oil content, Fe and Zn, and protein content, which is used in breeding groundnut varieties with improved nutrition. Identification and characterization of the genes involved in enhancing nutritional traits can further contribute to the efforts of biofortification in groundnut. Socioeconomic aspects related to the consumption of biofortified food are the major constraints to promote biofortified crops. This chapter will focus on nutritional value, genetics of nutritional quality traits, breeding approaches, and future strategies for biofortification of groundnut.

Keywords

Biofortification · Groundnut · Oleic acid · Oil content · Protein · Fe and Zn

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1 Introduction

1.1 Economic Importance of Groundnut

Groundnut or peanut (*Arachis hypogaea* L.) is commonly referred to as a poor person's nut and is an economically important oilseed and food crop of the world. It ranks third in area and second in production among the seven major legumes (common bean, cowpea, lentil, soybean, pigeon pea, chickpea) in the world (FAOSTAT 2018). It is cultivated in >100 countries and globally occupying a total area of 28.51 million ha and a total production of 46 million tons in 2018 (FAOSTAT 2018). China (17.39 mt), India (6.70 mt), Nigeria (2.89 mt), Sudan (2.88 mt), the USA (2.48 mt), Myanmar (1.60 mt), Tanzania (0.94 mt), and Argentina (0.92 mt) are the major groundnut-producing countries in the world (FAOSTAT 2018). Asia and Africa together constitute about 90% of world groundnut production. In the past decade, global groundnut area and production have marginally increased by 20% and 24%, respectively (Fig. 14.1) (FAOSTAT 2018). The groundnut consumption has grown at a rate of 2.53% in 2015 and will continue to rise in 2024 (Peanuts Market 2020). As the global food industry is shifting toward increased use of plant-based foods, peanuts with ~25% protein gain prominence as plant-based protein.

Groundnut is one of the important crops for global food security and to enhance small-holder farmer production systems (Toomer 2018). It is cultivated under rainfed conditions in Asia and Africa mainly by the small-holder farmers in semi-subsistence systems with no or limited irrigation and limited inputs other than land and labor (Janila et al. 2016). Due to its low moisture requirement and highly adaptive nature, groundnut is capable of producing modest yields under unfavorable conditions of climate compared to other crops (Ojiewo et al. 2020). Furthermore, it is

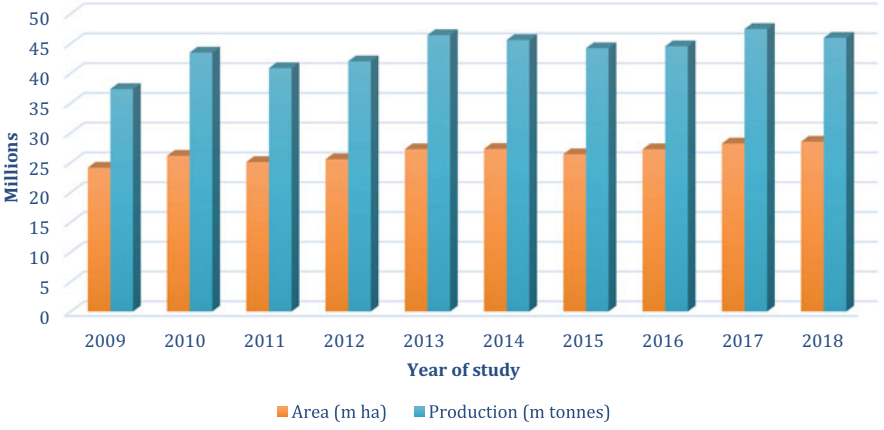

Fig. 14.1 Graphical representation of groundnut production over the past 10 years (2009–2018). (Source: FAOSTAT 2018)

a relatively low-input legume, which also enhances soil fertility through biological nitrogen fixation along with its ability to be a major food source.

1.2 Nutritional Value of Groundnut

Groundnut kernels contain 48–50% of fat, 25–28% of protein, 10–20% of carbohydrates, and are a rich source of dietary fiber, minerals, and vitamins (Pasupuleti et al. 2013). The nutritional profile of groundnut is presented in Fig. 14.2. Groundnut plays an important role in the world economy both as an oil and food crop. The major shift in consumption patterns, adoption of a healthy lifestyle by consumers, and the high nutritive value of groundnut supported the positive growth in the global market.

Groundnut oil is extensively used for cooking in countries like India, China, Myanmar, and Vietnam (Pasupuleti et al. 2013); however, there is a shift toward increased food uses of groundnut. The kernels can be consumed in different forms such as raw, boiled, or roasted form. Beyond these, groundnut products are widely used to produce confectioneries, groundnut butter, roasted groundnuts, snack production, soups, desserts, and extenders in meat products. The valuable components in its oil are also helpful in the manufacturing of baby products and pharmaceutical industries. The by-products of groundnut oil like oil cake and other crop residues such as straw and hay are extensively used as animal feed.

Groundnuts are mainly used for the processing of oil in many developing countries (Nautiyal 2002). It comprises about 50% monounsaturated fatty acids (MUFAs), 33% paraformaldehyde (PFAs), and 14% saturated fatty acids of the total fat profile (Feldman 1999). The highest MUFAs in the groundnut diet reduce total body cholesterol by 10% and bad low-density lipoprotein (LDL) cholesterol by 14% (Pelkman et al. 2004). It can be used for cooking and deep frying because of its

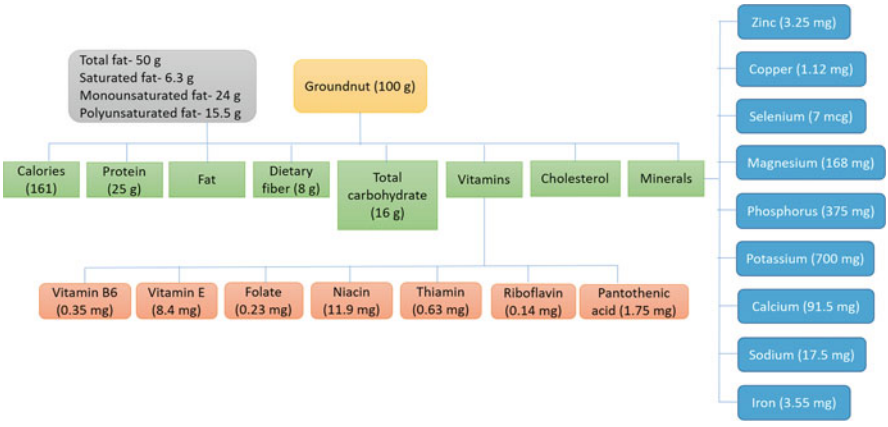

Fig. 14.2 Nutritional profile of groundnut. (Source: Agricultural Research Service (ARS), U.S. Department of Agriculture (USDA) 2018)

high smoking point (Singh and Diwakar 1993). It is also a valuable source of vitamins and helps in reducing malnutrition in developing countries. As per the recommended dietary allowance (RDA), consumption of 100 g of groundnut provides 75% niacin, 60% folate, 55.5% vitamin E, 53% thiamine, 35% pantothenic acid, 27% pyridoxine, and 10% riboflavin (Arya et al. 2016). This nutritional hub of vitamins helps in the fight against heart diseases, Alzheimer's disease, and is good for pregnant women. Groundnut oil cake obtained after extraction of oil is the richest source of protein and may reach up to 50% (Zhao et al. 2011). The lower sugar levels with a glycemic index (GI) of 14 in groundnuts help diabetic patients to reduce the blood glucose levels (Foster-Powell et al. 2002). It also contains a small amount of mineral composition that is essential for proper functioning and maintaining body health. Also, 100 g of groundnut could provide required RDA levels of minerals such as 127% copper, 42% magnesium, 84% manganese, 54% phosphorus, and 57% iron, which help in reducing the risk of metabolic syndrome and type II diabetes (Song et al. 2005). The resveratrol, a phenolic compound with potent antioxidant properties, occurs naturally in the skin, roots, and shell of groundnut ranging from 0.022 to 1.72 μg/g (Meredith and Anderson Alfred 2003; Francisco and Resurreccion 2008). It plays a vital role in preventing the risk of cardiovascular, cancer, Alzheimer's disease, and delays aging (Sales and Resurreccion 2014).

Groundnut is a rich source of protein than other nuts. The protein content in groundnut cake can reach as high as 50% and is used as feed for poultry and livestock (Zhao et al. 2011). All 20 amino acids are available in groundnut cake in variable proportions with "arginine" being the biggest source (USDA 2014). Owing to their high nutritional content, groundnut and groundnut-based food products are promoted as nutritional food supplements and in ready-to-use therapeutic foods (RUTFs) to combat energy, protein, and micronutrient malnutrition among malnourished children and women. Groundnut in the form of flour, protein isolates, and meal is desirable to prepare food supplements mixed with other grains, and the protein products are being tested to add nutritional value to the diets of children in Senegal. Groundnut kernel skin contains phenolics with high antioxidant properties.

1.3 Importance of Crop in Alleviating Micronutrient Deficiencies (MND) in the Face of Climate Change and Increasing Population

More than 2 billion individuals worldwide are assessed to be inadequate in vitamin A, iron, and zinc. Providing micronutrient-fortified foods to populations severely affected by MND is one of the means to avert the number of affected individuals. Biofortification is the process of improving the nutrient content of crops through various approaches like conventional breeding, development of transgenics, and mineral fertilization (Garg et al. 2018). Groundnut is considered a rich source of a wide range of nutrients and bioactive compounds with health benefits (Variath and Janila 2017). It is also a highly climate-resilient crop as it can tolerate mild moisture stress as well as heat stress. Countries like Asia and Africa with widespread MNDs

as well as the largest population growth are the major contributors to groundnut area and production (FAOSTAT 2018). Even with unpredicted rainfall patterns and temperatures hovering around the critical limit still, groundnut continues to dominate in these regions due to its resilience to abiotic stresses and highly adaptive nature (Variath et al. 2020).

As a food crop for home consumption, groundnut kernels are utilized in various forms: ground, boiled, roasted, paste, etc. It is common to see different forms of snack items and confectionery made from groundnut. Roasted groundnut, shelled and unshelled, is an important snack and pass-time food, particularly in Africa and Asia. In general, it is a very important ingredient in diets. Food products based on groundnut meet the key criteria of availability, affordability, acceptability, nutritional quality, and business interest to curb undernutrition (Anim-Somuah et al. 2013). Groundnut is widely used in the preparation of food supplements [ready to use supplementary foods (RUSF)] and RUTFs for fighting MNDs in infants and aged ones under different programs of United Nations Children's Fund (UNICEF) (Variath and Janila 2017). RUTF products are widely used to treat acute malnutrition, particularly among the vulnerable groups of children and women. Projects like "SPRING nutrition" in Ghana and "Groundnut Scaling" in Mali, Ghana, and Nigeria promote household consumption of groundnut for enhanced nutrition. In the United States Agency for International Development (USAID)-funded project, groundnut-based nutritional supplements were used to treat malnutrition in children of Bangladesh, highlighting the importance of this crop in overcoming MNDs.

To treat malnutrition in children, the United Nations Educational, Scientific and Cultural Organization (UNESCO) uses "Plumpy'Nut," a RUTF in Nigeria. Groundnut-based RUTFs, which is a mixture of groundnuts, sugar, oil, and milk powder, is a cost-effective solution to curb malnutrition in Malawi, Sierra Leone, and Ghana as a part of Project Peanut Butter (2020) (www.projectpeanutbutter.org), and currently UNICEF's RUTF program feeds 2.6 million children in Africa (UNICEF 2014). In Uganda, a sorghum–peanut blend (SPB) with honey, ghee, and energy content of about 430 kcal/100 g was used (Amegovu et al. 2013) to treat acute malnutrition in infants and young children. For infants, drinkable infant food with groundnut was developed to prevent and manage malnutrition. A blend was prepared using golden amaranth grain along with groundnuts, beans, sesame, and cowpeas (Nabuuma et al. 2013). Under the banner Plumpy Field Grain Legumes for Nutritional Security 1549, nine independent producers have formed a network in manufacturing nutritional products to treat and prevent malnutrition in endemic regions of the developing world (www.Plumpyfield.com). Plumpy'Nut as RUTF to address severe acute malnutrition and Plumpy'Sup as RUSF to address moderate acute malnutrition have been prepared.

2 Evaluation of Genetics Resources for Biofortification in Groundnut

The genus *Arachis* is classified into nine taxonomic sections, which are clearly distinguished based on morphological, cytogenetic features, cross–compatibility, and geographical distributions (Krapovickas and Gregory 1994). Among the members of the genus *Arachis*, four gene pools were identified based on sexual compatibility (Singh and Simpson 1994). The primary gene pool consists of two tetraploid species, *A. hypogaea* and *A. monticola*; secondary gene pool with diploid species from a section *Arachis* that are cross-compatible with A. *hypogaea*; tertiary gene pool contains the section *Procumbentes* species that are weakly cross-compatible with *A. hypogaea*; and the fourth gene pool represents the remaining wild *Arachis* species. All the sections have perennial species, except for *Arachis* and *Heteranthae*, which contain both annual and perennial species. Around 81 species were described (Valls and Simpson 2005), which includes both the diploids and tetraploids, of which the cultivated type is *A. hypogaea* L.

The cultivated groundnut presents considerable morphological variation and is classified into two subspecies *fastigiata* and *hypogaea* based on the arrangement of flowers on the main axis, branching pattern, growth habit, and the pod morphology (Krapovickas and Gregory 1994). The subspecies '*hypogaea*' has two botanical varieties, '*hypogaea*' (Spreading-Virginia runner and Semi-Spreading-Virginia bunch types) and '*hirsuta*' (Peruvian runner), whereas the subspecies '*fastigiata*' has four ('*fastigiata*'—*Valencia;* '*vulgaris*'—*Spanish bunch;* '*peruviana*'; and '*aequatoriana*') (Gregory et al. 1973; Krapovickas and Gregory 1994). There are three different market types; Virginia type (large-seeded) or runner type (small-seeded), Valencia type, and Spanish type.

Various accessions of groundnut are conserved globally at various international and national gene banks in India, China, Brazil, the USA, and also International Crops Research Institute for Semi-Arid Tropics (ICRISAT) (Ntare et al. 2006; Pandey et al. 2012). Most of these accessions have been characterized for various morphological, agronomical, biochemical, and nutritional traits using descriptors of groundnut (IBPGR and ICRISAT 1992; Jiang et al. 2006) and reported a large variations for quantitative, qualitative traits, seed quality traits (oil content, high oleic acid, high protein content, rich in micronutrients), and traits' resistance to abiotic and biotic stresses (Barkley et al. 2016). The variability parameters and heritability for the nutritional traits in groundnut are presented in Table 14.1.

Wild species of groundnut remain mostly untapped due to difficulties in growing, low pod yield, and the existence of crossability barriers with the cultivated spp. A study conducted with 72 wild *Arachis* accessions revealed that oil content variability was in the range of 51–63% across 3 years of trials, and accession of WH10026 of *A. rigonii* reported 61–62% oil content (Huang et al. 2012). The oil content in the normal cultivated species was reported as 40–55% (Huang et al. 2012). Among the three different growth habitat groups of groundnut, viz., Virginia runner, Virginia bunch, and Spanish bunch, the oil content varied from 45% to 52%, 47% to 52%, and 45% to 54%, respectively (Bansal et al. 1993; Raheja et al. 1987). In groundnut

Table 14.1 Variability and heritability of nutritional quality traits in groundnut

Trait	Material	Range (%)	PCV (%)	GCV (%)	Heritability (%)	Reference
Oil content	146 recombinant inbred lines (RILs) obtained from a cross TG26 × GPBD4	43.11–48.00%	Low (2.88)	Low (1.66)	High (66.9)	Sarvamangala et al. (2011)
	72 wild *Arachis* accessions	51.40–63.34%	–	–	–	Huang et al. (2012)
	14 M5 groundnut mutant lines along with their parents, Dacca-1 and PK-1	48.04–49.92%	Low (1.24)	Low (1.07)	High (74.12)	Rashid et al. (2012)
	147 F7-derived RILs from the cross TG-49 × GPBD-4	42.55–51.06%	Low (4.04)	Low (2.40)	Moderate (35.30)	Azharudheen et al. (2013)
	147 RILs from the cross TG 49 × GPBD 4 and their reverse crosses	42.90–49.68%	Low (3.24)	Low (2.41)	Moderate (55.50)	Krishnamurthy et al. (2015)
	160 advanced breeding lines	37–60%	–	–	High (90.00)	Janila et al. (2016)
	10 Virginia bunch groundnut genotypes	36.84–44.71%	Low (7.39)	Low (5.33)	Moderate (51.75)	Bhargavi et al. (2017)
	70 groundnut genotypes	44.07–56.16%	Low (8.04)	Low (3.84)	Low (22.85)	Chavadhari et al. (2017)
	12 advanced lines	47.49–61.66%	–	–	High (72.00)	Dolinassou et al. (2017)
	Advanced BC_2F_6 and BC_1F_8 generations of backcrosses	43.2–49.5%	Low (2.6)	Low (2.3)	High (79.8)	Patidar and Nadaf (2017)
	Interspecific derivatives involving *Arachis batizocoi*, *A. duranensis*, *A. cardenasii*, and *A. sps manfredi-5* groundnut	44.6–53.9%	(4.58)	(4.01)	High (76.5)	Bera et al. (2018)
	150 RILs from a cross CHICO × ICGV 12473	45.91–53.32%	Low (4.60)	Low (3.64)	High (62.57)	Saini and Sharma (2018)
	30 genotypes	45.15–49.05%	Low (2.20)	Low (2.12)	High (93.30)	Meghala Devi et al. (2019)

(continued)

Table 14.1 (continued)

Trait	Material	Range (%)	PCV (%)	GCV (%)	Heritability (%)	Reference
Protein content	146 recombinant inbred lines (RILs) obtained from a cross TG26 × GPBD4	23.44–34.50%	Low (6.35)	Low (4.92)	Low (49.1)	Sarvamangala et al. (2011)
	12 genotypes	18.05–20.30%	–	–	High (86.00)	Noubissié et al. (2012)
	147 RILs from the cross TG 49 × GPBD 4	24.40–31.59%	Low (6.32)	Low (5.39)	High (72.70)	Krishnamurthy et al. (2015)
	160 advanced breeding lines	19–31%	–	–	High (70.00)	Janila et al. (2016)
	US peanut mini core collection	20.6–30.4%	–	–	–	Wang et al. (2016)
	Advanced BC_2F_6 and BC_1F_8 generations of backcrosses	30.8–39.6%	Low (3.9)	Low (3.6)	High (85.5)	Patidar and Nadaf (2017)
	Interspecific derivatives involving *Arachis batizocoi*, *A. duranensis*, *A. cardenasii,* and *A. sps manfredi-5* groundnut	21.9–26.7%	Low (4.73)	Low (3.54)	Moderate (56.00)	Bera et al. (2018)
	150 RILs from a cross CHICO × ICGV 12473	21.68–26.80%	Low (7.97)	Low (5.87)	Moderate (54.17)	Saini and Sharma (2018)
	30 genotypes	24.10–26.60%	Low (1.12)	Low (0.95)	High (71.00)	Meghala Devi et al. (2019)
Oleic acid content	146 recombinant inbred lines (RILs) obtained from a cross TG26 × GPBD4	33.37–58.88%	(8.07)	(7.43)	High (84.6)	Sarvamangala et al. (2011)
	147 F7-derived RILs from the cross TG-49 × GPBD-4	45.22–55.84%	Low (3.98)	Low (3.65)	High (84.10)	Azharudheen et al. (2013)
	Advanced BC_2F_6 and BC_1F_8 generations of backcrosses	40.0–73.1%	Low (8.5)	Low (8.3)	High (95.5)	Patidar and Nadaf (2017)
	Interspecific derivatives	34.4–48.6%	Moderate (10.22)	Low (9.64)	High (88.9)	Bera et al. (2018)

	150 RILs from a cross CHICO × ICGV 12473	23.95–44.46%	Low (15.88)	Low (13.10)	High (68.00)	Saini and Sharma (2018)
Iron (Fe) and zinc (Zn)	184 genotypes of peanut mini core collection	Fe: 18.3–30.8 mg/kg Zn: 28.4–43.8 mg/kg	–	–	Fe: High (73.49) Zn: High (72.90)	Upadhyaya et al. (2012)
	64 groundnut genotypes	Fe: 33–68 mg/kg Zn: 44–95 mg/kg	–	–	Fe: High (81) Zn: High (92)	Janila et al. (2014)
	184 lines from $F_{2:3}$ generation from a cross ICGV 06099 × ICGV 93468	–	Fe: Low (12.60) Zn: Low (6.84)	Fe: Low (7.71) Zn: Low (4.07)	Fe: High (64.24) Zn: High (62.21)	Sadaiah et al. (2017)
	25 lines from F_2 and 75 lines from F_3 generations of Girnar-3 × FDRS-10	–	Fe: F_2 (28.35); F_3 (32.50) Zn: F_2 (52.85); F_3 (27.90)	Fe: F_2 (43.28); F_3 (45.97) Zn: F_2 (56.66); F_3 (40.57)	Fe: F_2 (42.90); F_3 (50.00) Zn: F_2 (89.00); F_3 (47.28)	Ajay et al. (2016)
	25 lines from F_2 and 75 lines from F_3 generations of TG-37A × FDRS-10	–	Fe: F_2 (29.97); F_3 (7.29) Zn: F_2 (34.65); F_3 (20.63)	Fe: F_2 (41.55); F_3 (45.05) Zn: F_2 (34.70); F_3 (20.74)	Fe: F_2 (80.17); F_3 (2.62) Zn: F_2 (89.62); F_3 (98.92)	Ajay et al. (2016)
	120 groundnut genotypes mini core collection	–	–	–	Fe: High (78.33) Zn: High (74.81)	Zhang et al. (2019)

mini core collection (Upadhyaya et al. 2003) and wild groundnut accessions (Upadhyaya et al. 2011), the variability of oil content was in the range of 45–55%. The oil content is 45–54% among the interspecific derivatives from *Arachis batizocoi*, *A. duranensis*, *A. cardenasii,* and *A.* Species *Manfredi-5* (Bera et al. 2018).

Among 160 cultivated elite breeding lines evaluated across six environments, the mean kernel protein content ranged from 15% to 31% (Janila et al. 2016). Significant variability in the kernel protein among groundnut accessions within the mini core collection revealed 20–30%, with an average of 26% (Wang et al. 2016). The variability for protein content varied from 18% to 20% among 12 groundnut genotypes studied (Noubissié et al. 2012). The protein content is 22–27% among the interspecific derivatives involving *Arachis batizocoi*, *A. duranensis*, *A. cardenasii,* and *A.* Species *Manfredi-5* (Bera et al. 2018).

Various studies showed significant variation in major (oleic, linoleic, and palmitic acid) and minor (arachidic, behenic, palmitoleic, and gadoleic) fatty acids among groundnut cultivars. A study involving fatty acid profiling of 174 groundnut genotypes revealed that oleic acid and linoleic acid together constitute about 80% of total fatty acid, and the remaining portion was occupied by other fatty acids and interestingly 19 genotypes contain myristic acid (Nawade et al. 2016). The oleic acid is 34–49% of the total fat among the interspecific derivatives involving *Arachis batizocoi*, *A. duranensis*, *A. cardenasii,* and *A.* species *Manfredi-5* (Bera et al. 2018). The oleic acid and linoleic acid were in the range of 43–87% and 5–33%, respectively, among nine groundnut cultivars in Uganda (Achola et al. 2017).

Very little work was done in groundnut for the variability of Fe and Zn concentration in seed (Janila et al. 2014; Lal and Singh 2007; Sadaiah et al. 2017; Upadhyaya et al. 2012). The Fe and Zn concentrations were in the range of 18–31 and 28–44 mg/kg, respectively, among 184 accessions of groundnut mini core collection studied (Upadhyaya et al. 2012). Likewise, in a study of 64 groundnut genotypes, the range of Fe in the seed ranged from 33 to 68 mg/kg and Zn in the seed ranged from 44 to 95 mg/kg (Janila et al. 2014).

3 Genetics and Breeding in Groundnut

3.1 Genetics of Nutritional Quality Traits

Understanding the genetic components, such as inheritance, gene action, number of alleles/genes, and genotypes × environmental interactions of nutritional traits, is needed for genetic improvement of these traits. The nutritional traits such as oil content, protein content, fatty acids content, and Fe and Zn content are reported to be quantitatively inherited. The published information on the heritability, genetic advance, and correlation among the traits aids in determining the effectiveness of selection and predicting the gain from selection and planning of suitable breeding strategies is given in Table 14.1.

The oil content in groundnut is governed by both additive and nonadditive gene action. The low heritability of oil content in cultivated species has been a setback for improving this trait (Reddy and Murthy 1996). The identification of stable sources of high oil content in high-yielding superior backgrounds (Janila et al. 2016) and the availability of robust and high-throughput phenotyping tools like near-infrared reflectance spectroscope (NIRS) enable improvement of oil content. Wild species with >55% oil content in their seeds are reported and can be used in the groundnut breeding programs (Huang et al. 2012; Nagraj and Murthy 1988). The variation in fatty acid composition is not only attributed to the genetic makeup of cultivar but also influenced by genotypic differences and environmental conditions during crop growth (Hassan and Ahmed 2012).

Norden et al. (1987) identified the first high oleate mutant line F435 with 80% oleic acid and 2% linoleic acid. Multiple works were carried out to decipher the inheritance pattern for oleic acid content and reported monogenic, digenic inheritance, duplicate recessive genes, and multiple allelic variations (Barkley et al. 2016; Lopez et al. 2001). The high oleic acid content in groundnut is controlled by two recessive genes located on A and B genomes (Knauft et al. 1993). The majority of works proposed a two-gene qualitative inheritance pattern (Lopez et al. 2001; Moore and Knauft 1989) and also quantitative inheritance especially in normal oleic groundnuts (Upadhyaya and Nigam 1994). The variations observed in high oleic trait segregation speculated the involvement of multiple allelic variations (Lopez et al. 2001), as well as epistatic interactions (Isleib et al. 2006).

Two genotypes, ICGV 06099 (57 mg/kg Fe and 81 mg/kg Zn) and ICGV 06040 (56 mg/kg Fe and 80 mg/kg Zn), are identified with a high concentration of Fe and Zn in their seeds and can be used as parents in the groundnut breeding program (Janila et al. 2014). Limited studies are reported in groundnut for improvement of protein content. The presence of high variability of protein content is also reported (Misra et al. 1992; Parameshwarappa et al. 2010) and the moderate variability reported among groundnut genotypes by Noubissié et al. (2012). Protein content varied from 17% to 25.2% and is inversely proportional to the seed size (Prathiba and Reddy 1994).

The coefficient of variation for oil content is low (Azharudheen et al. 2013; Dolinassou et al. 2017; Rashid et al. 2012; Sarvamangala et al. 2011; Saini and Sharma 2018). The low estimates of GCV and PCV for protein and oleic acid content indicate the role of environment on the expression of the trait among the groundnut genotypes (Saini and Sharma 2018). Similar low estimates of GCV and PCV for oil, protein, and oleic acid content are also reported by Patidar and Nadaf (2017). Meghala Devi et al. (2019) reported low estimates of PCV and GCV for protein and oil content among 30 groundnut genotypes.

Both high (Dolinassou et al. 2017; Janila et al. 2016; Rashid et al. 2012; Sarvamangala et al. 2011; Saini and Sharma 2018), moderate (Azharudheen et al. 2013; Bhargavi et al. 2017), and low heritability (Chavadhari et al. 2017) for oil content and moderate to high (Krishnamurthy et al. 2015) and low heritability (Sarvamangala et al. 2011) for protein content are reported. For protein content, high heritability along with low genetic advance as percent of mean suggests the

preponderance of nonadditive gene effects (Noubissié et al. 2012; Meghala Devi et al. 2019). High heritability is reported for oleic acid content with moderate genetic advance as percent mean, indicating the possibility of effective selection (Patidar and Nadaf 2017; Sarvamangala et al. 2011; Saini and Sharma 2018). High heritability for Fe and Zn (Ajay et al. 2016; Janila et al. 2014; Sadaiah et al. 2017; Upadhyaya et al. 2012; Zhang et al. 2019) indicates the possibility of genetic improvement for Fe and Zn.

Researchers reported significant G × E interaction for oil content among the groundnut genotypes studied (Azharudheen et al. 2013; Bansal et al. 1993; Baring et al. 2013; Barrientos-Priego et al. 2002; Dolinassou et al. 2017; Dwivedi et al. 2003; Isleib et al. 2006; Janila et al. 2016). Studies also reported significant G × E interaction for protein content (Janila et al. 2016; Sarvamangala et al. 2011) and Fe and Zn content in groundnut (Alake and Ayo-Vaughan 2017; Janila et al. 2016; Upadhyaya et al. 2012), suggesting the influence of the environment on these traits and the need for stability analysis to identify stable genotypes for these traits.

Negative correlation observed between protein content and oil content enables breeding of varieties with high protein and low oil content that adds value to the confectionary quality of groundnuts (Bera et al. 2018; Janila et al. 2016; Sarvamangala et al. 2011; Saini and Sharma 2018). Whereas oil content showed a positive correlation with pod yield (Saini and Sharma 2018), crop maturity (Baring et al. 2013) and oleic acid (Dwivedi et al. 1993), oleic acid and O/L ratio (Sarvamangala et al. 2011). In contrast, the oil content showed a negative association with oleic acid content and pod yield (Mercer et al. 1990; Yusuf et al. 2018). The protein content was negatively correlated with oleic acid content in the US groundnut mini core set studied by Wang et al. (2016). There is a positive correlation between Fe and Zn, which indicates the possibility of simultaneous improvement of both these micronutrients in groundnut (Janila et al. 2016; Sadaiah et al. 2017; Upadhyaya et al. 2012).

3.2 Breeding for Nutritional Quality

The breeding efforts targeting oil content and fatty acid composition in groundnut resulted in the commercialization of cultivars with (a) low oil content, (b) high oil content, and (c) high oleic content. High oleic cultivars were first commercialized in the USA, and since then high oleic cultivars are commercialized in Australia, Argentina, China, and India owing to the demand for shelf life benefits and consumer health benefits. The first groundnut variety with high oleic acid content was SunOleic 95R, derived from a cross between a high oleic breeding line (F435) and a component line of "Sunrunner" at the University of Florida. In 1997, another variety with improved characteristics over SunOleic 95R was released as SunOleic 97R. SunOleic comprises about 80% oleic acid along with 2–3% linoleic acid content, which improves the shelf life of groundnut products (Peanut Institute 1999). Improving high oleic traits is possible using a single seed decent (SSD) method based on phenotyping of the progenies in $F_{4/5}$ and marker assisted selection. The two mutant

alleles of *FAD2A* and *FAD2B* confer high oleic acid content of about 80%, and the bulks in $F_{4/5}$ generation are tested using NIRS to select high oleic progenies in the bulk-pedigree method. Marker assisted breeding uses Kompetitive allele-specific PCR (KASP), allele-specific markers, or SNPs to select the two mutant alleles (Huang et al. 2019).

After 8 years of efforts of the Indian Council of Agricultural Research-Directorate of Groundnut Research (ICAR-DGR), the State Agricultural Universities (SAUs) in India, and ICRISAT, the first high oleic acid varieties Girnar 4 (ICGV 15083) and Girnar 5 (ICGV 15090) were commercialized in India in 2019. The work was supported by the National Mission on Oilseeds and Oil Palm (NMOOP) of the Department of Agriculture, Co-operation and Farmers' Welfare (DoAC and FW) of the Government of India and OPEC Fund of International Development (OFID). High oleic lines are under various stages of testing for release in Bangladesh and Myanmar in partnership of ICRISAT with the Bangladesh Agricultural Research Institute (BARI) and the Department of Agricultural Research (DAR) in these two countries, respectively.

Based on extensive multilocation and multiyear testing, ICGVs 05155, 06049, 06041, 06420, and 03043 are identified as stable high-oil-yielding lines (Janila et al. 2016). The groundnut variety, GJG 32 (ICGV 03043), with high oil content released by Junagadh Agricultural University (JAU), Junagadh, Gujarat, in 2018 is gaining popularity in India. CGM-1 (ICGV 06420) was released for cultivation in 2020 in Chhattisgarh state of India, and it is selected from a cross between ICGV 87846 (resistant to rust and LLS) and ICGV 99240 (early maturity) followed by phenotyping of fixed lines in advanced generations. In Nigeria, high oil groundnut varieties like Samnut-21 (51%), Samnut-23 (53%), Samnut-24 (53%), and Samnut-25 (51.5%) were released recently (Ajeigbe et al. 2015). Three groundnut cultivars—Yuhua 4 (57.7%), Yuhua 9 (61.1%), and Yuhua 14 (59.3%)—with high oil content were released in China (Wang et al. 2020).

3.3 Phenotyping Methods

Phenotyping for nutritional traits involves destructive or nondestructive methods of estimation of groundnut kernels. Oil content is measured by the Soxhlet method, which estimates the solvent extracted oil from a grounded sample. The fatty acids can be estimated by gas chromatography (GC) using fatty acid methyl esters (Phillips and Singleton 1978). The seed protein content is determined by the Kjeldahl method that estimates the nitrogen content that can then be converted to protein content (Pasupuleti and Nigam 2013). This method involves digesting the sample in sulfuric acid to produce ammonium sulfate, followed by adding strong alkali like sodium hydroxide and the release of ammonia. The ammonia obtained is then captured by boric acid, and the amount of nitrogen is estimated by titrating boric acid with sodium carbonate. The protein content in the seed is expressed using estimated nitrogen content by using conversion factor 5.46 for groundnut. Though these destructive methods are accurate, it is cumbersome and time-consuming and

hence robust methods like Technicon Autoanalyser (Pulse Instrumentation Ltd., Saskatoon, SK) (Singh and Jambunathan 1980) for determining protein content, nuclear magnetic resonance (NMR) for oil content (Jambunathan et al. 1985), and NIRS (Misra et al. 2000) for oil, protein, and fatty acid content are used. Protein and oil/fat content are measured as a percent of kernel weight, and fatty acids are measured as a percent of the total fat/oil.

X-ray diffraction spectroscopy (XRF) and atomic absorption spectrometer (AAS) are two possible instruments to measure Fe and Zn concentration from groundnut kernels. The tri-acid method is generally used for the digestion of samples to determine Fe and Zn before measuring in AAS as per the procedure proposed by Sahrawat et al. 2002. One-gram sample is digested with a 10 mL tri-acid mixture of nitric acid, sulfuric acid, and perchloric acid in a 10:0.5:2 v/v ratio. After overnight cool digestion, the sample is again digested at 20 °C for 1 h followed by digestion at 230 °C for about 2 h to get a clear and colorless solution. After digestion tubes were cooled, the content was dissolved in water and diluted to 75 mL with distilled water. The final aliquot can be used to measure Fe and Zn under AAS, and results can be expressed as mg/kg. XRF is a nondestructive method to estimate Fe and Zn and can be more useful to screen a large number of genotypes and populations (Pasupuleti and Nigam 2013).

In the case of breeding programs, the improvement of quality traits is very intricate and challenging for the plant breeders as the samples are large in number, and the wet chemistry methods use destructive approach. Consequently, the phenotyping is delayed to advanced generation when the samples are less. The nondestructive methods like NIRS and XRF can reduce the time and efforts in phenotyping the nutritional quality traits and are cost-effective (Pasupuleti and Nigam 2013) compared to the destructive method. Deploying molecular markers if available in breeding also enables selection of the trait without phenotyping. The utilization of molecular markers has simplified the mapping of genes or QTLs and the identification of valuable alleles in the segregating populations for the nutrition quality traits, thus hastening the development of diagnostic markers for use in breeding.

4 Morphological and Molecular Diversity Analysis

In comparison to elite lines, landraces are still the preferred choice for diversity studies as they are considered to harbor useful alleles that can be exploited in plant breeding programs across the world (Dwivedi et al. 2016). As screening of large collections for the specific trait of interest is expensive and cumbersome (Holbrook and Stalker 2003), subsets like core and mini core collections of groundnut are established in China (Jiang et al. 2010), the USA (Holbrook et al. 1993; Holbrook and Dong 2005), and India (ICRISAT) that are representative of the genetic diversity for various traits of breeding interest and thereby facilitate easier access to the genetic resources. Literature on nutritional quality diversity in groundnut is limited, hence, there is a need to characterize germplasm for nutritional quality traits and

identify sources from the cultivated germplasm or wild species for use in nutritional quality improvement of groundnut.

Morphological and molecular diversity analysis is useful to characterize the germplasm collections and identify potential parents for a breeding program (Dwivedi et al. 2016). Five botanical types of cold-tolerant groundnut accessions are screened for 15 morphological traits, and oil and protein content using principal component analysis (PCA). Four clusters were formed with tall, larger leaflets, highest seed yield, large pod, and seed size based on nine principal scores. Accessions with higher seed oil content were present in the second cluster, and accessions with high seed protein content in the third cluster and fourth cluster. These accessions are superior to checks in their performance and hence can be used in breeding to develop high-yielding cultivars with improved nutritional quality (Upadhyaya et al. 2009).

Upadhyaya et al. (2011) evaluated 269 accessions of 20 wild *Arachis* species for morpho-agronomic traits, oil, protein, and total sugar content. Four clusters are formed based on the first five principal component scores. Annual types were grouped in clusters I and II, whereas all the perennials in clusters III and IV. *Arachis duranensis* showed a high diversity index, and the best 20 accessions with superior trait combinations were identified and used for subsequent introgression of unique alleles into cultivated groundnut. A mini core collection of groundnut germplasm lines is evaluated for nutritional, oil quality, and yield component traits using D^2 analysis that grouped the mini core collection into 15 clusters, of which 12 clusters had a single genotype each. Accessions in cluster I were low in oleic acid content and high in protein content, the majority of which were Spanish bunch types and cluster II accessions were of the Virginia type with high oleic acid content. Thus, oleic acid content ranked first (30.75%) followed by protein content (28.78%) in their contribution toward the divergence of genotypes (Mukri et al. 2014). Based on nutritional quality and agronomic traits, all the 64 advanced backcross lines are grouped into two major clusters. The first cluster consisted of only two varieties with low to medium oleic acid content, whereas the second cluster contains newly developed advanced backcross lines with high oleic acid. High oleate advanced backcross lines in second cluster can be used in future groundnut breeding programs to develop high-yielding varieties for quality traits (Gangadhara and Nadaf 2016).

In recent times, molecular markers have played a very crucial role in evaluating the genetic diversity across different crop species (Bhad et al. 2016; Milla-Lewis et al. 2010; Ren et al. 2014; Roomi et al. 2014). Their utilization in crop improvement programs has revealed the occurrence of low diversity (de Carvalho Moretzsohn 2004; Herselman 2003) and also moderate to high polymorphisms (Oteng-Frimpong et al. 2015; Roomi et al. 2014) within the cultivated types of groundnut. Wild *Arachis* accessions of 72 are phenotyped for oil content and genotyped using 136 genome-wide simple sequence repeats (SSR) markers. Population structure and phylogenetic analysis revealed three clusters, with *A. duranensis* exhibiting the highest diversity index of 0.35 and 129 unique alleles. Of which, three alleles are associated with higher oil content, which can be used as a potential for the future groundnut improvement program (Huang et al. 2012).

Samaha et al. (2019) evaluated the genetic diversity of five groundnut cultivars for seed quality, yield, and yield component traits using 20 RAPD primers. The dendrogram analysis revealed two main clusters, the first one included only one cultivar and the other cluster was divided into subclusters consisting of four cultivars. The genetic similarity matrix values based on RAPD markers ranged from 0.91 to 0.71, with Gregory and Giza-5 identified as the most distant among five groundnut cultivars. A total of 440 polymorphic bands are identified with an average of 2.99 and a gene diversity index of 0.11 in a study involving 196 groundnut cultivars grown across different regions of China screened with 146 highly polymorphic SSR markers (Ren et al. 2014). For different ecological regions, a neighbor-joining tree of cultivars is constructed showing a significant difference between cultivars from the south and the northern regions. The cultivars adapted to these regions revealed large genetic distance, indicating that there is distinct genetic differentiation among individual cultivars.

Cultivated groundnut has a narrow genetic base with limited variability due to single-event hybridization of diploid wild ancestors and genetic barriers to gene/allele transfer due to cross-incompatibility. Thus, the diversity assessment of the groundnut accessions for nutritional quality traits using morphological descriptors, molecular markers, the extent of geographical diversity, and the utilization of wild species would benefit the groundnut breeding program.

5 Brief Account of Molecular Mapping for Nutritional Traits/Grain Micronutrients Concentration in Groundnut

The establishment of molecular markers for quality traits will be useful in breeding. However, QTLs responsible for protein, oil content, and Fe and Zn content are less studied in groundnut. Few QTLs are identified for groundnut oil content in different genetic backgrounds. Recombinant inbred lines (RILs) developed from Tamrun OL01 and a Spanish (BSS 56) revealed one major QTL for oil content with marker PM36 using bulk segregant analysis with a phenotypic variance explained (PVE) of 11.03% (Gomez Selvaraj et al. 2009). A mapping population of 146 RILs from a cross, TG26 × GPBD4, is screened with more than 1000 SSR markers to identify four QTLs for oil content with the PVE ranging from 1.5% to 9.1% and two QTLs for protein content with PVE >10.0% (Sarvamangala et al. 2011). Pandey et al. (2014a) developed improved genetic map using S-population (SunOleic 97R × NC94022) and T-population (Tifrunner × GT-C20) with 206 (1780.6 cM) and 378 (2487.4 cM) loci and revealed six and nine QTLs controlling oil content from the S and T populations, respectively. The breeding efforts are directed toward enhancing the seed oil content along with desirable fatty acid composition for oil industry, and low oil content and high oleic acid for confectionary and food industry. Mapping of *fatty acid desaturase 2* (*FAD2)* genes revealed that these genes have a greater effect for oleic acid, linoleic acid, and O/L ratio, and no effect on total oil content (Pandey et al. 2014b).

Wilson et al. (2017) identified three QTLs in an advanced backcross population using 91 SSR markers for oil content. Khera et al. (2019) genotyped two AB-populations, viz., AB-pop1 (ICGV 91114 × ISATGR 1212) and AB-pop2 (ICGV 87846 × ISATGR 265-5A), and screened them using Diversity Arrays Technology (DArT) and SSR markers to predict the loci of oil content. Genetic maps are constructed with 258 and 1043 loci for AB-pop1 and AB-pop2 populations and identified two and six QTLs of oil content from AB-pop1 and AB-pop2 populations with a PVE of 9.6–14.8% and 7.4–52.5%, respectively. A RIL population (Xuhua 13 × Zhonghua 6) with 2595 loci revealed seven QTLs on five linkage groups for oil content, including the major and stable QTL *qOCA08.1* on chromosome A08 with a PVE of 10.14–27.19% (Liu et al. 2020).

A F_2 mapping population developed from ICGV 07368 × ICGV 06420 is screened for oil and oleic acid content with 854 marker loci to identify eight QTLs for oil content including two major QTLs, *qOc-A10* and *qOc-A02*, with a PVE of 22.11% and 10.37%, respectively, and three major QTLs for oleic acid content, namely, *qOle- A09-1* (A09), *qOle-A09-2* (A09), and *qOle-B09* (B09), explaining 17.4%, 34.2%, and 33.8% PVE (Shasidhar et al. 2017). A major QTL for protein and oil content, oleic acid, linoleic acid, palmitic acid, stearic acid, and behenic acid is identified from a RIL population of 432 lines derived from TMV 2 and its mutant, TMV 2-NLM, using genic and nongenic transposable elements in groundnut. The protein content explained PVE of 26.4% followed by oleic acid content of 15.1% using single marker analysis and QTL analysis (Hake et al. 2017). Limited literature is available on QTLs and associated markers for Fe and Zn in the groundnut. Sadaiah et al. (2017) identified putative genomic regions linked with kernel Zn and Fe concentration using the $F_{2:3}$ population derived from cross, ICGV 06099 × ICGV 93468. The SSR markers linked with Fe content are GM2638, IPAHM245, and SEQ9G05 with PVE of 1.75%, 2.25%, and 6.01%, respectively. The SSR markers linked with Zn content are SEQ1B09, IPAHM245, and SEQ9G05 with PVE of 0.23%, 2.19%, and 6.34%, respectively. The QTLs associated with the nutritional traits are summarized in Table 14.2.

6 Association Mapping Studies

Although several QTLs are identified for various traits of interest using large populations, bi-parental mapping relatively has a narrow genetic base due to the limited number of parents and recombination events. Compared to bi-parental genetic mapping, Genome Wide Association Studies (GWAS) offers a higher-resolution mapping to predict marker–trait associations followed by the discovery of candidate genes with advanced levels of genetic recombination across the population studied (Xu et al. 2016). So far, very few studies are reported on association mapping in groundnut and a comprehensive GWAS study is reported for agronomically important traits by Pandey et al. (2014a). Association analysis of 292 groundnut cultivars with 583 polymorphic SSR markers for oil content separated the entire population into two groups (Liu et al. 2020). Two associated

Table 14.2 Various QTLs of nutritional quality traits reported in groundnut

Traits	Population	QTLs	PVE (%)	Reference
Oil	Tamrun OL01 × BSS 56	1	11.03	Gomez Selvaraj et al. (2009)
	TG 26 × GPBD 4	4	1.50–9.10	Sarvamangala et al. (2011)
	SunOleic 97R × NC94022 Tifrunner × GT-C20	15	2.53–10.23	Pandey et al. (2014b)
	Zhonghua 10 × ICG 12625	1	14.36	Huang et al. (2015)
	Florunner × TxAG-6	13	2.00–18.00	Wilson et al. (2017)
	ICGV 07368 × ICGV 06420	8	5.60–22.10	Shasidhar et al. (2017)
	ICGV 91114 × ISATGR 1212 ICGV 87846 × ISATGR 265-5A	2 6	9.6–14.8 7.4–52.5	Khera et al. (2018)
	Xuhua 13 × Zhonghua 6	7	10.14–27.19	Liu et al. (2019)
Protein	TG 26 × GPBD 4	2	>10.0	Sarvamangala et al. (2011)
Oleic acid	ICGV 07368 × ICGV 06420	3	17.4–34.2	Shasidhar et al. (2017)
Fe and Zn	$F_{2:3}$ population derived from cross ICGV 06099 × ICGV 93468	3 markers	Fe: 1.75–6.01 Zn: 0.23–6.34	Sadaiah et al. (2017)

markers, namely AGGS1014_2 and AHGS0798, were identified for oil content in a RIL population with a PVE of 6.90–9.94% (Liu et al. 2020). For a better understanding of the oil synthesis process, transcriptome analysis of 49 groundnut cultivars revealed 5458 differentially expressed genes (DEGs), which include 2243 positive DEGs and 3215 negative DEGs (Wang et al. 2018). From GWAS, 48 significant insertion/deletion (InDel) markers were identified that are associated with seed oil content. Haplotype from candidate genes identified on A03 in an independent population showed variable alleles for oil content. This locus helps in the understanding of genetic control associated with the expression of oil content.

Zhang et al. (2019) reported the GWAS studies on 120 genotypes of a mini core collection of groundnut and identified one QTL of Zn with PVE of 12.54%. From this study, one SNP at position 22,450,498 bp on LGB05 significantly associated with Zn accumulation. Groundnut requires a relatively large collection of accessions for GWAS to detect meaningful associations due to its genome complexity. These association mapping studies offer insights into groundnut diversity and provide valuable information to groundnut breeders and geneticists toward variety improvement. They provide information about the relatively high degree of structure in the association panel and significant MTAs.

7 Genomic Tools for Biofortification

7.1 Structural and Functional Genomics Resources Developed

The recent developments and application of molecular tools in groundnut have resulted in elucidation and understanding of genes involved in important biosynthetic pathways such as oil content, fatty acid profile, and Fe and Zn content. However, the genetic and molecular mechanisms behind these trait variations are poorly studied.

Oil content is a complex polygenic trait, and several studies were conducted to elucidate pathways and identify candidate genes involved in lipid metabolism. Analysis of seed transcriptome in different developmental stages of high- and low-oil groundnut varieties identified 1500 unigenes involved in lipid metabolism and seven possible metabolic pathways involved in oil accumulation during seed development (Yin et al. 2013). In another study by Wang et al. (2018), transcriptome analysis of 49 groundnut cultivars identified a total of 147 gene clusters located in 17 chromosomes associated with oil content. Further, GWAS identified 48 significant insertion/deletion (InDel) markers associated with seed oil content, of which one InDel cluster located on the A03 chromosome was repeatedly found in three different environments. Glycerol-3-phosphate acyltransferase 9 (GPAT) is the main rate-limiting enzyme in the triacylglycerol (TAG) biosynthetic pathway and plays an important role in seed oil accumulation. From A and B genome of groundnut, two *Arachis hypogaea glycerol-3-phosphate acyl transferase 9* (*AhGPAT9)* genes were isolated with a similarity of 95.65% with 165 site differences (Lv et al. 2020). Further, an allelic polymorphism study of these genes on 171 groundnut germplasm revealed 64 haplotypes (*a1* to *a64*) for *AhGPAT9A* and 75 haplotypes (*b1* to *b75*) for *AhGPAT9B*. The haplotypes with high oil content were found to be *a5*, *b57*, *b30*, and *b35*, whereas *a7*, *a14*, *a48*, *b51*, and *b54* were low oil content types (Lv et al. 2020).

In the case of the fatty acid profile, very less attention is given to identifying the genes associated with high oleic acid formation. The majority of the works is on the fatty acid desaturase (*FAD*) gene involved in converting oleic acid to linoleic acid (Chen et al. 2010; Wang et al. 2015). The gene expression profile of high and normal oleic groundnut cultivars revealed that redox signaling acted as a messenger to connect the signaling transduction between the high-oleic content and *stearoyl-ACP (acyl carrier protein) desaturase 2 (SAD 2)* (*Aradu.XM2MR*), transcription level during seed development. The *SAD2* is an important gene in the fatty acid biosynthetic pathway that converts stearic acid into oleic acid (Liu et al. 2020). The gene *AhNRAMP1* is an iron transporter that is induced strongly in the roots under iron (Fe)-deficient condition in groundnut (Xiong et al. 2012). The genes *AhDMT1* (*Arachis hypogaea* Divalent Metal Transporter gene 1) and *AhIRT1* (Iron Regulated Transporter 1) are involved in iron transportation in groundnut (Shen et al. 2014; Xiong et al. 2012).

7.2 Genome Sequencing

The developments in low-cost high-throughput sequencing technologies have resulted in generating sequence information of several crops including groundnut. Cultivated groundnut is an allotetraploid that is derived from multiple hybridization events involving the two diploid progenitors *Arachis duranensis* (AA) and *Arachis ipaensis* (BB). Due to the close genetic relationship between the two diploid species, it is difficult to assemble the cultivated groundnut genome (Clevenger et al. 2016). Hence, to reduce complexity, both the diploid progenitors of groundnut were sequenced (Chen et al. 2016; Lu et al. 2018; Varshney et al. 2019) and the diploid information was used as a reference to assemble the tetraploid genome (Bertioli et al. 2016). Recently, the complete nucleotide sequence of cultivated groundnut was released (Bertioli 2018; Chen et al. 2019; Zhuang et al. 2019) and assembled by the international groundnut community. The US and international groundnut researchers have developed a resource database (www.groundnutbase.org), which provides information about molecular markers and QTLs for various traits of interest, genetic maps of diploid and tetraploid *Arachis* species, diploid genome sequence data, and *A. hypogaea* transcriptome data.

8 Recent Concepts and Strategies Developed

8.1 Gene Editing

Genome editing, also called as gene editing, is a technique wherein the DNA is inserted, deleted, modified, or replaced in the genome of a living organism. Gene editing targets the insertions to site-specific locations into a host genome as compared to random insertions that are followed by other genetic engineering techniques. The methods used for editing include the engineered nucleases like mega nucleases, zinc finger nucleases (ZFNs), single-stranded oligonucleotides, transcription activator-like effector-based nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system (Boglioli and Richard 2015; Carlson et al. 2012; Esvelt and Wang 2013; Puchta and Fauser 2013). Among these, CRISPR/Cas9 system has been widely deployed as a genome editing tool in a variety of organisms (Cai et al. 2020; Miki et al. 2018; Sun et al. 2015; Yin et al. 2017).

Gene editing is used to improve oleic acid content in groundnut using CRISPR/Cas9 technology to mutate the *ahFAD2* genes in groundnut protoplast and hairy root cultures (Yuan et al. 2019). Although this technique successfully indicated the site-directed gene editing using CRISPR/Cas9 technology, the validation of high oleic trait in the seeds of putative transgenic plants could not be confirmed. In another study, the function of nod factor receptor (NFRs) genes *AhNFR1* and *AhNFR5* is validated using CRISPR-Cas9 (Shu et al. 2020). They reported that both these genes are essential for nodulation in groundnut. The knock-out mutants of the *AhNFR5* gene are non-nodulating while the mutants with *AhNFR1* genes could still form

nodules after rhizobia inoculation. Research efforts are ongoing to deploy gene-editing tools to reduce allergens in groundnut (Brazelton 2015).

9 Genetic Engineering for Enhancement of Micronutrients

Groundnut is a tremendous source of dietary proteins and essential oils, but it is poor in methionine, an essential sulfur-containing amino acid (Venkatachalam and Sathe 2006), vitamin A, and contains less amount of iron (4.5 mg/100 g) and zinc (3.2 mg/100 g) (Arya et al. 2016). Genetic engineering tools are used to transfer genes/improve the expression of genes for biofortification traits from other crops to groundnut. Groundnut plants transformed with the *LEAFY COTYLEDON1* (*AtLEC1)* gene showed a 4–16% increase in oil content accumulation along with alterations in the fatty acid profile (Tang et al. 2018). The *AtLEC1* gene is expressed in a seed-specific manner in the groundnut genome driven by the NapinA full-length promoter/truncated 230-bp promoter. In another study, overexpression and antisense expression of *AhGPAT9* genes in groundnut transgenics reveal overexpression of the *AhGPAT9* gene, resulting in a 5% increase in oil content, while transgenic plants with the antisense construct showed an average of 7% decrease in oil content in comparison to the wild type (Lv et al. 2020). The GPAT is an important regulatory enzyme involved in the triacylglycerol biosynthetic pathway.

In the case of fatty acid content, the major focus of genetic engineering studies is to silence the *FAD2* genes involved in the conversion of oleic acid to linoleic acid. The use of sense and antisense constructs of *ahFAD2* resulted in downregulation of the *FAD2*, which led to an increase of 70% oleic acid content in the seeds of transformed plants compared with 38% in untransformed plants (Huang et al. 2008; Yin et al. 2007). To improve provitamin A or β-carotene content in groundnut, *phytoene synthase* gene (Zm*psy1*) from maize is fused with the constitutive promoter or the oil body-specific oleosin promoters and the transformed plants expressed 70-fold increased β-carotene levels compared to the untransformed controls (Bhatnagar et al. 2011). Similarly, groundnut plants transformed using the *Zea mays phytoene synthase 1* (*Zm psy1)* and tomato *β-lycopene cyclase* genes expressed higher β-carotene levels (0.75–5.5 μg/g) when compared to the untransformed controls (0.01–0.03 μg/g) (Bhatnagar et al. 2011).

The protein quality in groundnut is improved by integrating an *artificial storage protein x* (*ASP x*) gene encoding a protein linked to the KOZAK translational enhancer with 75% essential amino acids, leading to the increase of limiting amino acids like valine, tyrosine, phenylalanine, isoleucine, leucine, and methionine (N'Nan Affouande Sylvie et al. 2020). Likewise, genes coding for metal iron transporters such as *AhIRT1* and *AhNRAMP1* are induced in roots when groundnut is intercropped with maize under Fe-deficit conditions, and its expression in tobacco enhanced the Fe deposition and showed tolerant Fe deprivation (Xiong et al. 2012). The transgenic approaches would help to introduce the identified genes for Fe and Zn in groundnut for better mineral uptake (Gantait and Mondal 2018). So far, transgenic groundnuts have not been released for commercial cultivation owing to

the difficulties in plant regeneration by tissue culture techniques, selection of transgenic events, and socioeconomic, ethical, and legal limitations.

10 Nutrient Bioavailability, Enhancement of Promoters, and Reduction of Antinutrients

Groundnut is considered a functional food due to the presence of several health-enriching nutrients (Arya et al. 2016). The nutrient bioavailability depends on the nutrient composition, the form in which they exist, and the presence of antinutrients in the ingested medium. The term nutrient bioavailability refers to the effect of metabolic events on nutrient utilization (Schönfeldt et al. 2016). Fats, proteins, carbohydrates, and fibers, which are present in their most beneficial forms, comprise the major nutrient components in groundnut. Consumption of groundnuts can lower total body cholesterol by 11% and bad LDL cholesterol by 14%, while the good high-density lipoprotein (HDL) cholesterol was maintained with the reduction in triglycerides (Pelkman et al. 2004). The high proportion of monounsaturated fats in groundnut ensures that it is easily digested due to the presence of a single hydrogen bond that is easily broken (Feldman 1999). High oleic groundnuts expressing about 80% of oleic acid content have been developed, and their consumption can reduce LDL cholesterol, thereby reducing the risk of cardiovascular diseases along with improved overall digestibility (Buttar et al. 2005).

For groundnut proteins, the Protein Digestibility Corrected Amino Acid Score (PDCAAS) was estimated to be 0.70 out of 1, whereas for whole wheat PDCAAS is only 0.46, which indicates that the amino acid requirement of human and their ability to digest it are more in groundnut compared to wheat based on PDCAAS value (Arya et al. 2016). In vitro protein digestibility is higher for small- and medium-seeded varieties as compared to bold seeded varieties in groundnut, which could be due to the variation in protein fractions and protein inhibitors present in the varieties studied (Prathiba and Reddy 1994). Groundnut is a good source of insoluble fiber and small amounts of soluble fiber (Higgs 2003). There is very limited information on the effects of fiber on nutrient bioavailability. However, studies indicate that the fiber does not bind to nutrients to restrict their uptake. The small amount of soluble fermentable fiber in groundnut can improve the bioavailability of some minerals (Greger 1999).

Antinutritional factors reduce the bioavailability of nutrients through the formation of indigestible complexes by combining with proteins and minerals (Francis et al. 2002), resulting in micronutrient malnutrition and mineral deficiencies. The antinutrients are classified into heat-stable and heat-labile groups based on their response/sensitivity to high temperature (Gemede and Ratta 2014). The heat-stable group maintains its form, structure, and function even at high temperatures that include phytic acid, condensed tannins, alkaloids, and saponins, whereas the heat-labile group may lose its structure as it is sensitive to the high temperature that includes lectins, cyanogenic glycosides, protease inhibitors, and toxic amino acids (Thakur et al. 2019).

Table 14.3 List of antinutritional factors present in groundnut

S. no.	Antinutritional factors	Effect	Concentration (mg/g)	Reference
1.	Trypsin inhibitor	Retardation of growth reduces protein digestibility.	5.6	Embaby (2010)
2.	Protease inhibitors/α-amylase inhibitor	Substances reduce protein digestion.	–	Gemede and Ratta (2014)
3.	Phytic acid	Reduce Ca and Fe absorption.	0.95–1.76	Coulibaly et al. (2011)
4.	Lectins/ hemagglutinins	Prevent absorption of digestive end products in the small intestine. Hypertrophy and hyperplasia of pancreas.	0.14	Ahmed (1986)
5.	Saponins	High concentration can alter the integrity of intestinal epithelial cells and effects the absorption of vitamins A and E.	–	Gemede and Ratta (2014)
6.	Tanins	Inactivation of many digestive enzymes and decreases protein digestibility.	8.9	Embaby (2010)

The antinutritional factors present in groundnut include trypsin inhibitor, protease inhibitors, phytic acid, phyto-hemagglutinin, saponins-condensed tannin, and α-amylase inhibitor (Table 14.3) (Thakur et al. 2019; Wang 2016). Being a leguminous crop, groundnut contains phytic acid, a food inhibitor that chelates micronutrients and also interferes with the bioavailability of other nutrients. However, the concentration of phytic acid (0.95–1.76 g/100 g) in groundnut (Coulibaly et al. 2011) is much lower compared to other legumes such as soybean (1.00–2.22 g/100 g) (Schlemmer et al. 2009). These antinutrients can become toxic if present in excess in diet and lead to health issues by reducing the nutritional significance of foods. Hence, the focus of the research community shifted toward reducing the detrimental levels of antinutrients to prevent toxicity and its associated health problems. Therefore, various traditional food processing methods such as cooking, fermentation, autoclaving, soaking, roasting, and puffing were employed to reduce the antinutrients level (Samtiya et al. 2020; Thakur et al. 2019; Nwadi et al. 2020). These methods increase the digestibility of proteins and mineral absorption, also ensuring the quality and safety of groundnuts for human consumption.

There are around 32 different types of storage proteins found in groundnut kernels, of which Ara h1, Ara h2, Ara h3, and Ara h6 exhibit allergen properties with an ability to cause life-threatening reactions upon consumption (Krause et al. 2010; Ojiewo et al. 2020; Pele 2010). The conclusive evidence for the occurrence of these allergen genes in the groundnut genome is supported by studies on the reference genome of the diploid A genome (*A. duranensis*, accession PI475845)

and transcriptome studies in few seed development stages (Chen et al. 2016; Clevenger et al. 2016).

To identify the five major allergens in the groundnut, a monoclonal antibody-based ELISA protocol was issued at ICRISAT to accelerate the screening of accessions for low allergen content (Pandey et al. 2019a). Several emerging techniques like oral desensitization, anti-IgE therapy, probiotics, soy-based immunotherapy, cellular mediator, engineered allergen immunotherapy, plasmid DNA immunotherapy, bacterial adjuvant, immune-stimulatory sequence, and oligodeoxynucleotide-based immunotherapy are in the initial stages to get approval for practically preventing the groundnut allergy in sensitive individuals. However, efforts directed at increasing tolerance among kids would prove to be a more successful approach. Groundnuts identified with low allergen content would be an important alternative approach in battling the allergenicity in sensitive individuals.

11 A Brief Account on Social, Political, and Regulatory Issues

One of the greatest advantages of the biofortification in crops is its acceptance by farmers and consumers to improve nutrition and contribute to the health and well-being of the masses. The ethical issues associated with biofortification are intertwined with things related to food safety, food sovereignty, aesthetic preferences, protection of natural resources, and localized food systems. These concerns can be overcome by public awareness of the various benefits and risks associated with the consumption of biofortified crops.

Biofortification has an immense potential in developing nutritious crops, which will serve as a promising tool for improved human health. The impact across social groups (e.g., women, children, elderly, rural populations), especially among those who are most vulnerable to micronutrient deficiencies, should be identified and addressed. Besides, the research efforts should be devoted to evaluating the combinations of the farmer and consumer-oriented traits by incorporating other micronutrient strategies and assessing multibiofortification approaches (De Steur et al. 2012, 2014; Joy et al. 2017).

In several countries like the USA, China, India, Australia, and Argentina, guidelines are put in place for testing and commercialization of high oleic groundnut varieties that have about 80% oleic acid content owing to their health benefits and shelf life benefits. Regulatory guidelines enable trading and processing of groundnut commodities with high nutritional value, for example, the high oleic groundnuts. At the 23rd session held on 25 February to 1 March, 2013, at Malaysia of the Codex Committee on Fats and Oils of the Joint FAO/WHO Food Standards Program (Codex Alimentarius 2013), Argentina proposed an amendment to the fatty acid composition of fats and oils to specifically recognize the high oleic groundnut with about 80% oleic acid content of total fat in the trade. In India, efforts are underway to develop guidelines to ensure the genetic purity of high oleic varieties in both seed and commodity value chains to ensure the supply of high-quality high-oleic commodities to the processing.

RUTFs are cost effective, safe, and best means to reduce severe malnutrition. Worldwide production of RUTF increased radically due to the development of manufacturing units and ventures at underdeveloped and developed countries. As global nutrition switches toward obesity and metabolic dysfunction, use of RUTF-manufactured and commercialized products is necessary to treat malnutrition (Bazzano et al. 2017). Around 5.2 million groundnut RUTF packets are unlocked until this year by UNICEF to fight against severe acute malnutrition.

Aflatoxins are potent carcinogens produced by the fungi *Aspergillus flavus*, which poses a serious health threat to humans and livestock as long-time exposure to aflatoxin is carcinogenic and causes liver problems (Valery et al. 2018). The permissible level of aflatoxin for human consumption in groundnut is 4 parts per billion (ppb) in the European Union, 20 ppb in the USA, and 30 ppb in India (Pandey et al. 2019b). Regulatory guidelines in developing countries to contain the aflatoxin in peanut-based foods are needed to ensure food safety of domestic consumption.

Groundnut is believed to be a potent allergenic food, which affects 1–2% of the world population (Valcour et al. 2017), and the highly affected countries are Australia, the USA, Canada, Denmark, France, and UK (Sicherer et al. 2010; Sicherer and Sampson 2014). The allergies may lead to ethical and social concerns in the widespread use of peanut-based foods. However, in Asia and Africa, which account for 90% of groundnut production, groundnut allergies are negligible.

12 Future Perspectives

Groundnut is a major source of nutrition offering numerous benefits from plant to seed level. Various studies have revealed the consumption of groundnut linked with improved human health and reduced risk of life-threatening diseases. In developing countries of Africa and Asia, the consumption of groundnuts enhances the nutrition status of the malnourished population. Owing to the high nutritional quality of groundnut and groundnut-based food products, and their affordability, availability, acceptability, and business interest that are the key criteria to reduce malnutrition in children and women, they are promoted as food supplants and RUTFs in developing works.

Further improving the nutritional quality of groundnut contributed to improved health and well-being of the communities in Asia and Africa where it is a part of the diet. The development of biofortified groundnut varieties such as high oil and oleic acid content, high iron and zinc, and vitamins will help to fight micronutrient deficiencies at the global scale alongside health benefits. With new tools in crop breeding, the nutritional quality of the cultivars can now be improved by deploying genomic tools and robust phenotyping tools. The reduction of antinutrients using processing technologies will mitigate malnutrition issues.

Thus, an integrated approach of biofortification strategies can improve human health through the consumption of micronutrient-fortified food products. Meanwhile, the ethical and safety issues of biofortified crops should be analyzed before making them available to the consumers. In this background, the success of the

biofortification programs is directly associated with improved policies including nutrition education, marketing, agricultural policy, and, finally, public awareness. Sometimes, the enrichment of micronutrients and vitamins will harm the color and taste of the end product and may not be liked by the consumers. Therefore, biofortified crops will have to have acceptable sensory, cooking qualities for greater adoption. Furthermore, a more systematic step toward developing biofortified crops, along with suitable agronomic management options, is needed to eliminate the micronutrient malnutrition in humans and ensure food and nutritional security.

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Vegetable Biofortification: An Underexploited Silver Lining for Malnutrition Management

15

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Abstract

Roughly 1000 million tonnes of vegetables enter the food chain of people across the globe. These are the cheapest and most readily available source of energy and nutrition. Vegetables except few starchy ones are rich in micronutrients compared to staple foods like cereals. Deficiencies of the 'big five', i.e. iron, calcium, iodine, selenium, and vitamin A, affect the health of half of the global population. It is not only the population of developing countries but also the developed countries face mineral deficiencies. Apart from supplementation in the forms of pills, people now want to move ahead from pill-popping society to natural products for the betterment of the health. This had led to the emergence of the science of biofortification, through various means: i.e. metabolic engineering (transgenic), agronomic biofortification, and genetic biofortification. Even after extensive research in the staple crops, proportionate success was not apparent as evident from the golden rice programme. The regulatory hurdles (transgenic approval) and narrow genetic base have pushed the biofortification beyond staple crops. The vegetables are inherently rich in minerals, antioxidants, and vitamins. These vegetable crops offer a wide range of variability in terms of the number of choices of crops across the seasons. Leafy vegetables are found to be one of the richest sources of iron and calcium. Coloured vegetables offer a wide choice to consumers along with anthocyanins and β-carotene. Promoting the knowledge of vegetable vis-à-vis biofortified crops should be included in the government's agenda nutritional programme, because of their potential to reach malnourished rural populations, who may have limited access to supplements and commercially fortified foods.

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Biofortification · Vegetables · Vitamins · Minerals · Nutrition

1 Introduction

Hidden hunger via mineral malnutrition has emerged as a major sector of malnutrition in human beings. Addressing malnutrition is a major challenge for agriculturists and nutritionists throughout the world. For the last several decades, researchers had focused on the improvement of yield. The recent study shows that focus on handful of crops has not only reduced the diversity available with the farming community but also today's food contains lower levels of iron, zinc, protein, calcium, vitamin C, and other nutrients than in the past (Marles 2017). Even after a successful green revolution throughout the world, the reports of the United Nations Food and Agriculture Organization (FAO) claim that around 821 million people are undernourished, while two billion are malnourished (FAO 2018). The deficiency of 'big five', viz. iron, calcium, iodine, selenium, and vitamin A, affects the health of half of the global population (Saeid et al. 2019).

Vegetables are considered essential for well-balanced diets since they supply vitamins, minerals, dietary fibre, and phytochemicals. Each vegetable group contains a unique combination and amount of these phytonutriceuticals, which distinguish them from other groups and vegetables within their own group (Table 15.1). In the daily diet, vegetables have been strongly associated with improvement of gastrointestinal health, good vision, and reduced risk of heart disease, stroke, chronic diseases such as diabetes, and some forms of cancer. Some phytochemicals of vegetables are strong antioxidants and are thought to reduce the risk of chronic disease by protecting against free radical damage, by modifying metabolic activation and detoxification of carcinogens, or even by influencing processes that alter the course of tumour cells. All the vegetables may offer protection to humans against

Table 15.1 Sources of nutrients from vegetables

Nutrients	Vegetables
Carbohydrate	Sweet potato, potato, cassava
Protein	Pea, lima bean, French bean, cowpea
Vitamin A	Carrot, spinach, pumpkin
Vitamin B1	Tomato, chilli, garlic, leek, pea
Vitamin C	Chilli, sweet pepper, cabbage, drumstick
Calcium	Hyacinth bean, *Amaranthus*, spinach beet
Iron	*Amaranthus*, spinach beet, spinach, lettuce, bitter gourd
Phosphorous	Pea, lima bean, taro, drumstick leaves
Vitamin B5	Spinach beet, *Amaranthus*, bitter gourd, pointed gourd
Iodine	Tomato, sweet pepper, carrot, garlic, okra
Sodium	Celery, green onion, Chinese cabbage, radish

chronic diseases. Nutrition is both a quantity and a quality issue, and vegetables in all their many forms ensure an adequate intake of most vitamins and nutrients, dietary fibres, and phytochemicals which can bring a much-needed measure of balance back to diets contributing to solve many of these nutrition problems. The promotion of healthy vegetable products has coincided with a surging consumer interested in the healthy functionality of food. Because each vegetable contains a unique combination of phytonutriceuticals, a great diversity of vegetables should be eaten to ensure that individual's diet includes a combination of phytonutriceuticals and to get all the health benefits. This article makes a review and discusses the nutritional quality and health benefits of the major groups of vegetables. More interdisciplinary work is required that involves nutritional and food scientists as well as others from biomedical fields to ascertain the through function of specific phytonutriceuticals.

Biofortification is the process by which the nutrient density of food crops is increased through conventional plant breeding, and/or improved agronomic practices and/or modern biotechnology. It is recognized as a nutrition-sensitive-agriculture intervention that can reduce vitamin and mineral deficiency. Biofortification is a conceptually simple strategy which requires no change in legislation but requires translational research from the agriculture and nutrition sectors. Biofortification can be achieved through three main strategies, viz. metabolic engineering (through transgenics), genetic biofortification (plant breeding), and agronomic biofortification. Agronomic biofortification and genetic biofortification follow the model of the soil-plant-consumer system, while metabolic engineering suffers from regulatory hurdles. In the developing countries, the major efforts of biofortification are focused towards the enrichment of staple food crops; however, very little effort has been made towards the biofortification of vegetables and fruit crops (O'Hare 2015). A major programme targeting staple crops for improvement of iron, zinc, and vitamin A was carried out through HarvestPlus to remove the specific deficiencies (HarvestPlus 2014; Bouis and Saltzman 2017). According to WHO (2018), it is well known that a diverse diet including vegetables, fruits, legumes, nuts, and whole grains provides all the nutrients needed for good health. Several vegetable crops have multiple times mineral than the cereals (Broadley and White 2010); however, rather than promoting these vegetables globally, the research focus has primarily been on the notable cereals and its biofortification (GRAIN 2019). It is also known that increasing the concentration of minerals in the crop may not translate into increased absorption in the gut. Antinutritional factors, like phytate, oxalates, saponins, nitrate, and anti-vitamins, etc., play important role in the absorption and bioavailability of the nutrients. The question of bioavailability is always associated with the nutrients in general and minerals in particular. Therefore, during biofortification, bioavailability should also be given equal importance. The biofortification strategy could radically reverse malnutrition if adopted and accepted by different populations (Steur et al. 2010; De Steur et al. 2015; Mogendi et al. 2016). Biofortification has the potential to address the nutritional challenges by improving the micronutrient content of commonly consumed foods. Further, the success of biofortification will depend on the synergistic collaboration between the health and agriculture sectors (Hotz and

McClafferty 2007). Efforts are underway around the world to create demand for high-yielding biofortified crops and to develop healthy food products from biofortified crops so that non-farmers and urban consumers can also benefit. Biofortified crops are now grown and consumed by more than 20 million people. Iron biofortification of beans, cowpea, and pearl millet; zinc biofortification of maize, rice, and wheat; and provitamin A carotenoid biofortification of cassava, maize, rice, and sweet potato are currently underway and at different stages of development (Bouis et al. 2011; Saltzman et al. 2013).

A lot of diversity is found for edible vegetables, and around 1097 vegetables are grown and consumed worldwide. Vegetables are the inexpensive sources of nutrients, along with the potential to generate on- and off-farm income to their growers. India is blessed with a huge diversity of vegetables; also it is the second largest producer of vegetables across the globe. In India, the share of vegetable accounts for 59% of the total horticultural produce. The Indian vegetable farming output has achieved its target for the production of vegetables, exceeding the daily recommended amount of 250 g of vegetables, by availability of 380 g per capita (Sagar et al. 2020). These foods also provide significant amounts of dietary fibre that helps to improve digestive function and lower the risk for high cholesterol, heart diseases, obesity, and diabetes. These are the storehouse of natural vitamins (vitamin A, folate, vitamin C, etc.) and minerals, such as iron (Fe), zinc (Zn), selenium (Se), iodine (I), and potassium (K), which act as antioxidants that help to limit cell damage from free radicals. An increase in vegetable consumption reduces the risk of cancer by 15%, cardiovascular diseases by 30%, and mortality by 20% (Rimm 1996), by increasing the availability of antioxidants such as ascorbic acid, vitamin E, carotenoids, lycopene, polyphenols, and other phytochemicals (Prior and Cao 2000). Vegetables can be a strong agent in malnutrition management due to several reasons like part of every household diet, more than 1000 million tonnes vegetables produced globally, in association with good health as discussed above, dietary diversity (1097 types), more bioavailability, etc. as discussed by Singh et al. (2020). Apart from high mineral content in the vegetables, nutraceutical-rich vegetables are now gaining importance that has started acquiring its space in the mind of consumers of developed countries. Vegetables are an excellent source of nutraceuticals also, and several nutraceuticals are reported to be found in vegetables. We, in this chapter, have tried to summarize the major efforts of biofortification in vegetable crops.

2 Improvement of Vegetable Crops Through Biofortification

2.1 Folate Biofortification

Plants are a major source of vitamin folate (vitamin B_9) for humans because they lack the ability to synthesize this vitamin. Deficiency of which can lead to several defects in newborn like neural tube defects (Berry et al. 1999; Geisel 2003), megaloblastic anaemia (Li et al. 2003), birth defects, impaired cognitive

Table 15.2 Folate composition per 100 g of various staple foods and vegetables (μg per 100 g FW)

Food crop	Folate (μg)	Food crop	Folate (μg)
Sweet corn, white corn	46	Plantain	22
Yellow corn	19	Mung bean	625
White rice	9	Lentil	180
Wheat flour	26	Chickpeas	172
Wheat bread	85	Soybean	165
Potato	16	Spinach	146
Cassava	27	Broccoli	108

development (Seshadri et al. 2002), increased risk of cardiovascular disease (Van Oort et al. 2003) and cancer (Choi and Mason 2000; Finglas et al. 2006). Besides, folates play a central role in the biosynthesis and metabolism of nucleotides, amino acids (serine, glycine, histidine, and methionine), and pantothenate (vitamin B5) (Blancquaert et al. 2010). Folates are stored in the liver, where the highest concentration is found; from there, it is distributed to the other body part and found in the most of the tissues and fluids (Hercberg and Galan 1992). The scientific concern is growing about folic acid supplementation and fortification, because its high intake could have adverse effects on human health, for instance, an increased risk of prostate and colorectal cancer (Cole et al. 2007). Moreover, its high dose may compromise the effectiveness of anti-folate drugs used in treatment against cancer, rheumatoid arthritis, and psoriasis (Arabelovic et al. 2007). The production and consumption of folate-rich food sources are the ideal way to prevent folate deficiency (Blancquaert et al. 2014). While conventional breeding is limited by the extent of variation for folate content in a crop (Table 15.2), metabolic engineering offers a rapid and high gain in folate content with limited variation (Blancquaert et al. 2014). Green leafy vegetables and legumes are a rich source of this vitamin, as are the fermented products. The daily recommended dietary allowances for folate are 400 μg/day for adults and 600 μg/day for pregnant women, respectively (Strobbe and Van Der Straeten 2017).

Tomato and potato had been the choice of vegetable for folate biosynthesis because of its worldwide acceptability as a food crop. The use of bacterial GTP cyclohydrolase-1 (*gch1*) in the plant was first achieved in *A. thaliana* and then subsequently in tomato following the same model. The *gch1* increases the *pterin* biosynthesis with a concomitant enhancement of the folate level (Hossain et al. 2004). The metabolic pathway engineering had changed the folate content on an average by twofold (Díaz De La Garza et al. 2004). Further, supplementation of P-aminobenzoate (PABA) in these transformed plants increased the folate content by tenfold (Díaz De La Garza et al. 2004).

In another study, De La Garza et al. (2007) showed that combining *gch1* and amino deoxychorismate synthase which catalyses the synthesis of pteridine and PABA, respectively, increased the folate content in the ripe fruit by 25-fold than controls. The animal-based, i.e. chicken *gch1*, was used to transform the lettuce

plant, and an increase of 2.1- to 8.5-fold higher folate content was recorded (Nunes et al. 2009). While engineering potatoes and *Arabidopsis* for higher folate content, Blancquaert et al. (2013) found that only engineering of pterin and PABA pathways are insufficient in increasing the folate content in the tubers. This example suggests that the two gene systems are insufficient in producing high folate plants in potato. We must look for other steps in the folate biosynthesis pathway and could be one of the reasons for not having a successful example of folate biofortification in many crops except rice and tomato. Two mitochondrial channelling enzymes were proposed to limit the flux: first, the bifunctional hydroxymethyldihydropterin pyrophosphokinase/dihydropteroate synthase (HPPK/DHPS), which performs the condensation of both precursors in mitochondria; second, in the last step of the pathway, folylpolyglutamate synthase (FPGS), which could exert a pulling effect (Waller et al. 2010; Gorelova et al. 2017). By targeting a total of four genes, De Lepeleire et al. (2018) transformed potato with *GTPCHI*, A*DCS*, *HPPK/DHPS*, and *FPGS* genes and successfully achieved the satisfactory level of folate in the tuber. They were able to enhance the folate content in potato by 12-fold (1925 mg/100 g dry weight), along with its stability under long-term storage.

The potato has a narrow range of folate diversity in the cultivated background, which ranges from 400 to 1300 ng/g folate on dry weight basis (Goyer and Navarre 2007). The same group further analysed the natural variation concerning the folate content in wild accessions also. The potato genotypes including the cultivars, landraces, and wild relatives were screened covering a wide diversity from the south, Central America, and Southern USA. The folate content as much as four times to that of commercial cultivars was identified (Goyer and Sweek 2011). In the similar study, Robinson et al. (2015) explored an even larger set of potato line including wild and cultivated. They found that there is about tenfold variation in the cultivated line and *Solanum tuberosum* subsp. *andigenum*, *Solanum vernei*, and *Solanum boliviense* that have the potential to produce more than double the folate concentrations of commercial cultivars, such as Russet Burbank.

Spinach is one of the best-known sources of folate among the vegetables. The concentration of which varied from 50 to 175 μg/100 FW of a leaf (Shohag et al. 2011). Also, the folate composition varies among the accessions. The major folate forms in spinach are H_4-folate, 5-CH_3-H_4-folate, 5-HCO-H_4-folate, and 10-CHO-folic acid. Out of these, 50% accessions showed the presence of 5-CH_3-H_4-folate (Shohag et al. 2011). In a strategy to further enhance the level of folate in the spinach, different molecules were tested which can boost the folate biosynthesis. Targeting this, Watanabe et al. (2017) practically demonstrated the effective way of folate biofortification using three candidate molecules, i.e. glutamic acid, magnesium, and phenylalanine. Normal growth of spinach was observed after phenylalanine and Mg^{2+}, whereas growth reduction was observed in glutamic-acid-added plants. The changes in the folate content ranged from twofold (306 μg in 100 g of fresh spinach), 1.4-fold, to no change over control, after the addition of phenylalanine, glutamic acid and Mg^{2+}, respectively. Thus, phenylalanine can be practically used for folate biosynthesis in these crops. Further analysis of intermediate

molecules of folate synthesis suggests that phenylalanine increased the level of pteridine and p-aminobenzoic acid (Watanabe et al. 2017).

Another important part of folate biofortification is its stability if its content is severely affected by storage and processing. Indeed, folates are unstable compounds, susceptible to oxidative and photo-oxidative catabolism (Scott et al. 2000), and degradation by pH variations as most folates are stable at pH 4–8 at 37 °C, except THF and dihydrofolate (De Brouwer et al. 2007). Blancquaert et al. (2010) suggested a few approaches to improve folate stability which include (1) engineering towards the accumulation of a more stable compound, (2) simultaneous accumulation of compounds with a protective mode of action (e.g. antioxidants such as ascorbate), (3) engineering compound salvage and breakdown reactions, and (iv) protein complexation.

2.2 Provitamin A Biofortification

Plants are the major source of provitamin A (carotenoids) for humans that produce mainly four types of carotenes, i.e. α-carotene and β-carotene accumulate in greater amounts than γ-carotene and β-cryptoxanthin that serve as the precursor of provitamin A. Carotenoids play an important role in human health (Fig. 15.1). The RDA of vitamin A is 700 mg retinol equivalents per day (Jiang et al. 2017). Among the several vegetables, potato is the most important staple source of food after the cereals and has a relatively very low content of provitamin A carotenoids. After the successful utilization of bacterial genes in the development of golden rice, a group of researchers successfully incorporated a similar system into potato for enhancing the carotenoid content (Diretto et al. 2007). They introduced the *Erwinia*-derived three genes encoding *phytoene synthase* (*CrtB*), *phytoene desaturase* (*CrtI*), and *lycopene beta-cyclase* (*CrtY*) under the control of constitutive promoter into potato. The carotenoids increased approx. 20-folds, to 114 μg/g dry weight and β-carotene 3600-fold to 47 μg/g dry weight in the tuber of the transgenic plants, i.e. golden potatoes (Diretto et al. 2007). They further characterized the carotenoid metabolites and transcripts. They also identified several endogenous genes as key regulators in carotenoid biosynthesis (Diretto et al. 2010). Carotene-rich cauliflower was developed through marker-assisted backcross breeding in which the orange (*OR)* gene was transferred into the genetic background of normal cauliflower. A variety was also released for commercial cultivation having a content of 8–20 ppm β-carotene (Kalia et al. 2018). The *OR*gene is the major regulator of carotenoid accumulation with other physiological roles in plants. The transgenic tomato when transformed with the *Arabidopsis* wild-type *OR* ($AtOR^{WT}$) and a 'golden SNP' containing *OR* ($AtOR^{His}$), different physiological changes were observed. The *OR* genes started chromoplast formation at a very early stage of fruit development and stimulated carotenoid accumulation at all developmental stages. It was also found that the plastid size was increased in the transformed plant. Moreover, *AtOR* overexpression promoted early flowering, fruit set, and seed production (Yazdani et al. 2019).

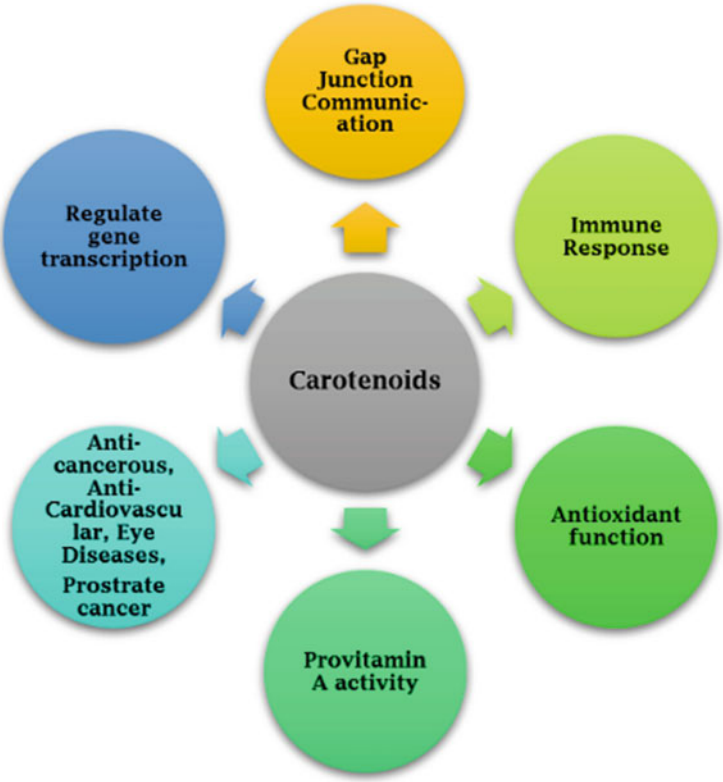

Fig. 15.1 Summary of health benefits of carotenoids

Carotenoid composition in pepper causes variations for fruit colour. A range of fruit colours is demanded by the consumer. The most frequent colour for pepper is red; however, yellow, orange, and green colours are also available (Devi and Sood 2019). During EMS mutagenesis studies of red pepper variety, some orange-coloured fruiting plant was observed. The analysis showed that a transition of A to G at 709 nucleotide position of β-*carotene hydroxylase*-2 gene was responsible for the altered phenotype. Further, analysis showed that this mutant had higher carotenoid content and serves as the natural source of biofortified pepper (Borovsky et al. 2013).

Carrot is one of the best dietary sources of provitamin A carotenoids for humans with a high β-carotene to retinol conversion ratio (Mills et al. 2008). It is well established that a modest amount of provitamin A from plants can maintain adequate vitamin A status (Dosti et al. 2006; Mills et al. 2007), and carrots of all colours, except white, supported vitamin A status equally well (Mills et al. 2008). The consumption of carrot is steadily taking pace because of its recognition as an important source of antioxidants, anti-cancerous, and vitamin A precursor (Dreosti 1993; Speizer et al. 1999). In general, the concentration of provitamin A in this

vegetable ranges from 6000 to 54,800 μg/100 g (Simon and Wolff 1987). Provitamin A carotenoids impart colour to foods; therefore, biofortification with these carotenoids will change the colour of crops. Biofortification of cassava with the provitamin A carotenoid, β-carotene is a potential mechanism for alleviating vitamin A deficiency. Cassava is a staple food in the African diet. Studies have shown that carotenoid bioavailability in food is low, with the bioconversion rate of β-carotene estimated to be as low as 12 mg to 1 mg retinol (Yeum and Russell 2002). However, biofortified cassava enriched with provitamin A carotenoids has successfully maintained vitamin A status in Mongolian gerbils (Howe et al. 2009). Thus, more information is required on the bioavailability and bioconversion of carotenoids from cassava in human subjects. The provitamin A bioavailability generally increases with the addition of fat (Yeum and Russell 2002).

Evidence for provitamin A-rich crops is more difficult to interpret as provitamin A carotenoids are first absorbed in the body and then converted into the active form of vitamin A according to the body's need for the nutrient. There is broad evidence (both efficacy and effectiveness) that provitamin A biofortified orange sweet potato reduces vitamin A deficiency in children in Mozambique (Low et al. 2007; Hotz et al. 2012a), Uganda (Hotz et al. 2012b) and in South Africa (van Jaarsveld et al. 2005), with an additional study in Bangladesh showing increased provitamin A concentration but not vitamin A status (Jamil et al. 2012). A study with provitamin A biofortified yellow cassava in Kenya showed an increase in vitamin A status and a greater increment in provitamin A concentrations in school children (Talsma et al. 2016).

To date, only a small provitamin A cassava efficacy study has been completed in Eastern Kenya with 5–13-year-old children. This trial demonstrated small but significant improvements in vitamin A status, measured both by serum retinol and β-carotene, in the yellow cassava versus the control group (Talsma et al. 2016).

2.3 Iodine (I) Biofortification

Iodine is the essential trace element required for human health in general and thyroid gland for secretion of thyroid hormone in particular. The deficiency of which causes neurocognitive defects, goitre, and thyroidism (hypo and hyper) (Zimmermann et al. 2015; Delshad and Azizi 2019). Only exogenous source can meet the requirement of this mineral (Vought and London 1967; Clar et al. 2002). The term iodine deficiency disorders (IDD), introduced by Hetzel (1983), has transformed the world's understanding of the problem, which leads to disorders ranging from endemic goitre to numerous other conditions. Throughout the world, this deficiency affects millions of human populations (Zimmermann and Andersson 2012). Hypothyroidism during pregnancy leads to preeclampsia, gestational diabetes, gestational hypertension, and spontaneous abortion (Stagnaro-Green 2011). Major sources of iodine supply are through fortified salt, oil, bread, and water (WHO 2007; de Benoist et al. 2008). At the recommendation of WHO, all the food grade salt should be fortified with iodine to control the IDD. The 80% of iodine in human body and animal originally come

from edible vegetable food under nature conditions (DeLong et al. 1997; Welch and Graham 2005), and the bioavailability of iodine in food can achieve as much as 99%. Storage of iodine in the plant tissues preferably occurs in vegetative tissue than to the reproductive tissue (Mackowiak and Grossl 1999). Thus, the leafy vegetables are more suitable for iodine accumulation (Dai et al. 2004), and its transport is mainly regulated through xylem (Zhu et al. 2003). The iodine fortification was achieved through agronomic biofortification in spinach (Zhu et al. 2003) and lettuce (Blasco et al. 2008; Voogt et al. 2010). However, Blasco et al. (2011) raised caution against the use of different forms of iodine during agronomic fortification. They used iodide (I^-) and iodate (IO^{3-}) in the nutrient solutions for growing lettuce. It was found that IO^{3-} is more suitable as a substrate for fortification than the I^-. The use of I^- increases the oxidative stress, while IO^{3-} produced the enzymes involved in the ROS detoxification (Blasco et al. 2011). In an in vivo study using different iodine biofortified vegetables, viz. potatoes, tomatoes, carrot, and lettuce, it was found that there is an increase in the urinary iodine content (UI; a way to measure iodine content in the body) among the individual consuming it (Tonacchera et al. 2013). While carrying out the human nutritional-based studies using the iodine biofortified legumes (Mogendi et al. 2016), it was found that among the participants of the East Africa knowledge of iodine, iodine-health link, salt iodization, and biofortification were very low, albeit lower at the household level. The participants also do not recognize iodine and biofortification as nutrient and novel approaches, respectively. After the study, the participants were ready to pay premium price for the biofortified products (Mogendi et al. 2016). Table 15.3 summarizes the iodine content of different plants in their edible parts after agronomic iodine biofortification. Gonzali et al. (2017) extensively reviewed the iodine biofortification strategy and suggested that higher gains in a shorter time can be achieved in terms of iodine content, through agronomic biofortification as compared to conventional breeding and metabolic engineering. Among the agronomic methods, the hydroponically grown crops showed highest rate of accumulation. They also suggested that leafy vegetables are the best-suited candidates for biofortification followed by tomato and potato (Gonzali et al. 2017). In carrot, similar soil-less and field experiment was carried out for iodine biofortification using foliar spray to plants. Three doses of iodine concentration were used, i.e. 0 mg/L, 50 mg/L, and 500 mg/L KIO_3, as a treatment for carrot. Under field condition, the carrot was able to accumulate triple and double amount of iodine than control at 500 mg/L and 50 mg/L, respectively (Signore et al. 2018). Using potassium iodate (KIO_3^-) as source, four brassica genotypes were grown under hydroponic conditions with three doses of iodine (0, 0.75, and 1.5 mg/L) to biofortify with iodine. Highest content of 66 μg/100 g FW was achieved using 1.5 mg/L of KIO_3^-. The 100 g of leafy vegetable was able to supply 24% of the iodine RDA (Gonnella et al. 2019).

Apart from inorganic sources of iodine, organic sources are also available and utilized for biofortification. Such one natural source of iodine is from marine algae, especially kelp (*Laminaria japonica* Aresch), a brown seaweed that can accumulate iodine and other minerals (potassium, magnesium, and iron) in high concentrations. The concentration of iodine in *L. japonica* may reach 734 mg/kg FW (Teas et al.

Table 15.3 Potential of different crops to accumulate iodine in their edible parts under different agronomic setups for iodine biofortification

S. No.	Crop	Plant part	Iodine content (mg/kg)	Reference
Hydroponic system experiment				
1.	Rice	Seed	1.3–8 mg/kg DW	Mackowiak and Grossl (1999)
2.	Spinach	Leaf and root	25–1800 mg/kg DW	Zhu et al. (2003)
3.	Lettuce	Leaf and root	60–800 mg/kg DW	Blasco et al. (2008)
4.	Water spinach	Shoot and root	600–1200 mg/kg FW	Weng et al. (2008c)
5.	Chinese cabbage	Edible part	5–100 mg/kg FW	Weng et al. (2008a)
6.	Tomato	Fruit	454–2423 μg/100 g FW	Caffagni et al. (2012)
Pot experiment				
7.	Spinach	leaf	0.1–50 mg/kg FW	Dai et al. (2004)
8.	Water spinach	Shoot and leaf	0.02–8 mg/kg FW	Dai et al. (2004)
9.	Cucumber	fruit	1–9 mg/kg FW	Weng et al. (2008b)
10.	Radish	root	1–13 mg/kg FW	Weng et al. (2008b)
11.	Chinese cabbage	Edible part	10–130 mg/kg FW	Weng et al. (2008a)
12.	Lettuce	Edible part	0–70 mg/kg FW	Hong et al. (2008)
13.	Potato	tuber	272–6245 μg/100 g FW	Caffagni et al. (2011)
14.	Tomato	fruit	3900–5375 μg/100 g FW	Caffagni et al. (2011)
Field experiment				
15.	Wheat	grain	7–18 μg/100 g FW	Ren et al. (2008)
16.	Potato	Tuber	2–89.4 μg/100 g FW	Caffagni et al. (2012)
17.	Tomato	Fruits	0.6–144 μg/100 g FW	Caffagni et al. (2012)
18.	Spinach	Leaf	5–22 mg/kg FW	Weng et al. (2013)
19.	Cabbage	Edible part	10–32 mg/kg FW	Weng et al. (2013)
20.	Chinese cabbage	Edible part	1–60 mg/kg FW	Weng et al. (2013)
21.	Egg plant	Fruits	0.3–1.2 mg/kg FW	Weng et al. (2013)
22.	Radish	Edible part	1–8 μg/100 g FW	Lawson et al. (2015)

2004). This kelp and diatomaceous earth was used for growing vegetables like Chinese cabbage, spinach, and radish (Weng et al. 2013). Following the application of algal iodine fertilizer at a rate of 12 mg m^{-2}, the average iodine concentration in the edible parts of cabbage, spinach, potherb mustard, Chinese cabbage, coriander, and celery was 9.1, 1.8, 5.8, 4.2, 19.3, and 9.4 mg/kg, respectively (Weng et al.

2014). When this fertilizer was applied at a rate of 75 mg m^{-2}, the average concentration of iodine in the edible parts of eggplant, hot pepper, cucumber, tomato, and long cowpea was 15.56, 21.30, 10.48, 7.74, and 8.42 mg/kg, respectively (Weng et al. 2014). Iodoacetic acid is also a form of organic iodine which can be sued to biofortify plants. In the study conducted by Weng et al. (2008c), when the fertilizer was applied at rates from 0.05 to 0.1 mg/dm^3, the iodine concentration in spinach leaves was higher than the following application of inorganic forms of iodine. Among the other organic sources tested, viz. 5-ISA (5-iodosalicylic acid), 3,5-diISA (3,5-diiodosalicylic acid), 2-IBeA (2-iodobenzoicacid), 4-IBeA (4-iodobenzoic acid), and 2,3,5-triIBeA (2,3,5-triiodobenzoic acid) were the potential candidates in the tomato biofortification study. All the sources except 2,3,5-triIBeA did not affect the plant growth.

Comparisons were also made among the method of biofortification of iodine. Whether direct soil application or foliar application is more suitable for achieving biofortified crops. In the study conducted in this aspect, according to Lawson et al. (2015), the soil-applied iodate or iodide has the potential to supply iodine for a limited extent of time, while the foliar application has a profound and long-time effect for iodine supply. It was also found that iodate is more suitable as an iodine source substrate in comparison to KI. It was also found that the desirable level of iodine in the lettuce and kohlrabi was obtained at 7.5 kg/ha $IO3^-$ in soil application whereas 0.5 kg/ha I^- as foliar application (Lawson et al. 2015). This study concludes that the foliar application is readily usable, as the quantity of iodine needed, and ease of application are major factors.

With the rise in iodine biofortified vegetables, a pertinent question rises that does these vegetables retain iodine upon cooking? Limited reports are available about the cooking methodology and iodine retention in the cooked foods. According to studies of Comandini et al. (2013) on carrot, potatoes, and tomatoes, it was observed that, when potatoes were cooked through boiling, there was no significant difference among the raw and cooked potato for iodine content. Similarly, baking of potatoes does not affect the iodine content. However, a significant reduction in the iodine content was observed in carrot after boiling. In the case of tomato, the iodine content significantly varied among raw and pasteurized puree as well as whole tomato (Comandini et al. 2013). On the line of same study, Caffagni et al. (2011) reported that the boiling of potato reduces 65% of the iodine, while the baking increased the iodine content to 165% as compared to raw potato. In the study using iodine biofortified potato, it was observed that significant loss of iodine occurs during boiling of dumpling and baking of vegetable pie, whereas no iodine loss was detected during baking of focaccia bread. However, the final cooked products contained as much as 33.3–52.7% of daily recommended intake in adults per servings (Cerretani et al. 2014). In the first-ever bioavailability study from biofortified carrot, normal, raw, and controlled cooked biofortified carrots are fed to Wistar rats (Pitkowska et al. 2016). It was concluded that a significantly high amount of iodine was detected in urine, faeces, and selected tissues of rats fed with raw carrots as compared to normal and controlled cooked carrot. The raw carrots can significantly increase the tri-iodothyronine concentration in the animal groups.

However, highest thyroid-stimulating hormone level was found in the animals fed with controlled cooked carrot. These convincing findings suggest that biofortified raw and controlled cooked carrot can be a potential crop for biofortification in any population to control iodine deficiency (Pitkowska et al. 2016). In the bioaccessibility study, Li et al. (2018a) first biofortified celery and pakchoi with iodine, then the leafstalk was soaked in the water for 8 h. They reported that the iodine loss rate of the biofortified celery was 3.5–10.4% only. More than 80% of the iodine in the biofortified celery was retained after cooking under high temperature. The highest bioaccessible iodine (BI) of the biofortified vegetables after digestion in simulated gastric and intestinal juice amounted to 74.08 and 68.28%, respectively. These studies suggest that high BI of vegetables provided a sound reference for the promotion of iodine biofortification as a tool to eliminate the IDD. Generally, boiling reduced iodine content, while steaming increased or left it unchanged, depending on genotypes (Gonnella et al. 2019).

As discussed above, iodine has important role in functioning of thyroid gland production which, through respective hormones, ensures the proper condition of an entire organism. The major three of the iodothyronine deiodinases (D1, D2, D3) are Se-dependent enzymes, i.e. there is a direct relationship between I and selenium (Se) (Bianco and Kim 2006). Fortifying crops with these two elements will provide a comprehensive tool in managing the thyroid-related problems. In order to achieve this goal, a study in lettuce was conducted by Smoleń et al. (2016a) where the group not only targeted the biofortification of these two elements but also tested whether the salicylic acid has role in channelling the uptake in plants or not. Smoleń et al. (2015) revealed that the introduction of salicylic acid (SA) into the nutrient solution (at a dose of 7.24 m MSA) improved the efficiency of I biofortification of tomato fruits. SA contributed a 157% and 37% increase in iodine accumulation in fruits for KIO_3 + SA and KI + SA, respectively. In another study, Smoleń et al. (2019a) biofortified six varieties of lettuce for both I and Se. They found highest concentration of 292.3 mg/kg (dry weight basis) iodine and 10.8 mg/kg Se (dry weight basis). Among the varieties, the accumulation of I was 10–30 times higher than Se. The same group of researchers successfully achieved combined biofortification for Se and I in carrot. They achieved the I and Se contents in roots increased 7.7 times for I and 4.9 times for Se as well as the average I:Se molar ratio was 0.28:1. Taking 100 g of biofortified carrot will sufficiently meet out the RDA of I and Se (Smoleń et al. 2019b).

2.4 Selenium (Se) Biofortification

Selenium is an essential element and constituent of protein in animals. At least 25 human proteins and enzyme glutathione are known to contain the selenium (Rotruck et al. 1973; Kryukov et al. 2003). The symptom of selenium deficiency includes cardiovascular diseases, bone and joint diseases in children, hypothyroidism, and lowered immune responses (Combs 2001; Rayman and Rayman 2002; Gupta and Gupta 2002). The extra intake of selenium is reported to be

anti-cancerous. In human trials, an intake of 250 μg/day reduced the cancer associated with liver, colon, oesophagus, and stomach (Whanger 2004; Combs Jr 2005). As such, the World Health Organization (WHO) has recommended the dietary allowance of ∼55–200 μg Se/day for adults (Wu et al. 2015), and the Institute of Medicine (USA) has suggested a tolerable upper intake of 400 μg Se/day for adults (White 2015). Se compounds from garlic and broccoli had cancer preventative effects, as these plants accumulate methylated amino acid derivative methylselenocysteine (MeSeCy) form inorganic Se (Ip et al. 2000; Finley and Davis 2001; Unni et al. 2005). A gene, *selenocysteine methyltransferase* (*SMT*) was cloned from the Se hyperaccumulator *Astragalus bisulcatus* (Neuhierl et al. 1999) and transformed into *Arabidopsis* and *Brassica* (Pilon-Smits and LeDuc 2009). Later, the same gene was transformed into *Nicotiana tabacum*, and it was shown that this gene can be utilized for nutritional improvement purposes (McKenzie et al. 2009). The same group also developed transgenics in tomato for production of MeSeCy by overexpression of *SMT* gene. They found that the accumulation of MeSeCy occurs in fruits but not in the leaves. It was also found that this MeSeCy was heat stable which can be readily used in tomato juice. Greater accumulation of MeSeCy was found when selenate was used (Brummell et al. 2011).

Higher accumulation of selenium was also observed in lettuce after the application of selenate as compared selenite (Ramos et al. 2011). Variation in selenium accumulation of at least twofold was observed among the lettuce germplasm in response to the Se application. The variation in accumulation among the genotypes is found to be associated with the differential expression of genes involved in the selinium/sulphur assimilation, and also a synergistic relationship was observed in the accumulation of Se and sulphur (S) (Ramos et al. 2011; Winkel et al. 2015). However, most of the worker has reported an antagonistic relationship among the accumulation of S/Se at high dose of selenate (Zayed et al. 1998; White et al. 2004; Lyi et al. 2005). Some studies show that a lower concentration of selenate promotes the accumulation of S (White et al. 2004; Lyons et al. 2005; Lefsrud et al. 2006). In general, selenate is less toxic than selenite to the plant growth (Ramos et al. 2011). Compared to earlier approaches where individual mineral was the target of biofortification, Smoleń et al. (2016a, b) successfully biofortified carrot with two minerals simultaneously. He used selenium and iodine fertilization to the plants. No negative effect on yield was observed after combined fertilization. The mineral content present in the edible part can easily supply the RDA for Se and I (Smoleń et al. 2016b). The plants parse does not require selenium for its growth and maintenance; however, inorganic form of selenium is converted to selenoamino acids and their derivatives. In an effort to enrich the garden pea with selenoamino acids, Garousi et al. (2017) carried out a pot experiment under a series of selenite doses in soil. They analysed the selenoamino acid content of shoot, pods, and seeds. It was found that highest increase was observed in the shoots followed by pods and then seeds. Among the amino acids, selenomethionine represented 65% of the total selenium content in shoots but was lower in pods and seeds (54 and 38%, respectively). The 3 mg/kg soil of selenium was found to be appropriate not only for overall growth of the plants but also for total protein accumulation (Garousi et al. 2017). In

turnip, the foliar application of selenite (50–100 mg/L) will be able to improve the selenium content in the roots. Apart from selenium accumulation, also a positive effect was observed in the accumulation of other minerals including magnesium, phosphorus, iron, zinc, manganese, and copper. Se foliar application was also able to increase the synthesis of protein and multiple amino acids instead of crude fat and total carbohydrate, thus improving the total nutrient status (Li et al. 2018b). Se biofortification was also achieved in mushroom *Cordyceps militaris* a vegetable with edible and medicinal properties. *C. militaris* is a highly nutritious ascomycetous fungus which is one of the most popular edible and medicinal mushrooms worldwide, especially in Asian countries, including China, Korea, Japan, and Singapore (Sung et al. 2007). An experiment conducted by Hu et al. (2019) under artificial cultivation, where five Se concentrations (0, 5, 10, 20, and 40 μg/g) and three forms of Se (selenate, selenite, and selenomethionine), was used in culture media. Compared with the control treatment, Se applications (40 μg/g selenate and selenite) significantly increased the Se concentration in fruiting bodies by 130.9 and 128.1 μg/g, respectively. Apart from increasing the Se, among the treated cultures, the concentration of cordycepin and adenosine was also increased. Se biofortification did not affect the yield of fruiting bodies. In the recent review, Newman et al. (2019) raised caution against the Se biofortification in crops, because Se may negatively impact the uptake of some essential minerals such as Ca, Mg, K, Fe, and Cu. Biofortification for Se should be focused on plants that are not dietary staples to avoid imbalances in the intake of other minerals, while other antioxidant compounds, such as phenols, anthocyanins, vitamin C, and flavonoids, increase with Se biofortification, making it suitable for vegetable fortification. Se biofortified vegetable microgreen in crops like coriander, green basil, purple basil, and tatsoi was successfully grown under hydroponic conditions (Pannico et al. 2020).

As most of the selenium is added in the soil from inorganic sources, the organic sources are also of prime importance for production of Se biofortified crops (Bañuelos et al. 2016). It was reported that Se hyperaccumulator *Stanleya pinnata* has potential as organic amendment in the soil (Bañuelos et al. 2015). During further studies in carrot and broccoli, Bañuelos et al. (2016) reported that *S. pinnata* can successfully produce Se biofortified carrot and broccoli after 3–4 years of soil amendment. This organic amendment also has no negative effect on the population of microbial biomass, arbuscular mycorrhizal fungi and actinomycetes (Chander and Joergensen 2007; Bañuelos et al. 2016). Similarly, Se-enriched plant materials were also tested as organic amendment (Banuelos et al. 1992; Ajwa et al. 1998). These seleneferous organic amendments can release the Se for crops for 2–3 years after adding. More than 80% of the Se remained in the soil even after two croppings of canola and fescue (Ajwa et al. 1998). Other studies have reported that plants absorb Se more rapidly from organic sources of Se compared to inorganic forms of Se (Kikkert and Berkelaar 2013). Shallot (*Allium cepa* L. *Aggregatum* group) was inoculated with both organic Se (selenocysteine) and inorganic Se (sodium selenite) along with the arbuscular mycorrhizal fungi (AMF) formulate inoculation to achieve Se biofortification. Selenocysteine showed the best effect on the growth and yield of mycorrhized plants, whereas sodium selenate was the most effective on

the non-inoculated plants. The soluble solids, total sugars, monosaccharides, titratable acidity, and proteins attained higher values upon AMF inoculation. Other minerals were also high in the bulb of the AMF-inoculated plants, i.e. Ca, Na, S, and Cl were higher in concentration than control. The AMF inoculation increased the bulb selenium content by 530%, and the Se biofortification with selenocysteine and sodium selenate increased this value by 36% and 21%, respectively, compared to control plants. Also, higher antioxidants activity and ascorbic acid were found in the bulb of AMF-treated plants. Thus, AMF offer a good choice as the input for Se biofortification along with an increase in overall quality of the produce (Golubkina et al. 2019). Naturally, Se-rich soil can also be used to grow the crop, and Se biofortification can be achieved (Bañuelos et al. 2020).

2.5 Zinc (Zn) Biofortification

The transportation of zinc from root to shoot through xylem occurs via transporter proteins. The same transporter, i.e. HMA4 and P_{1B}-ATPase, can transport metals like zinc (Zn), iron (Fe), and cadmium (Cd) (Courbot et al. 2007; Hanikenne et al. 2008) to the xylem. A high expression of HMA4 in the hyperaccumulator leads to high shoot concentration of Zn/Cd in the shoot (Hanikenne et al. 2008). However, the ectopic expression of 35S::AtHMA4 in tobacco resulted in increased Zn level in the shoot but decreased Cd level. This modification in the transport can be suitable for biofortification purpose (Siemianowski et al. 2011). This same construct was used to transform the tomato plant to check its efficacy as potential candidate for Zn biofortification. It was found that the transformed tomato had higher Zn content with respect to wild-type plants (Kendziorek et al. 2014). A study was conducted by Weremczuk et al. (2016) in soil and hydroponics media using the transformed tomato carrying *AhHMA4p1::AhHMA4* genes to determine whether mineral composition affects the translocation of these nutrients specially Zn and Cd. They reported that the expression pattern of cross homoeostasis gene (*LeIRT1*, *LeChln*, *LeNRAMP1*) changes in transgenics in medium-dependent fashion. Further, when the plants were grown in the soil with/without Cd, more efficient translocation of Zn was observed in the transgenics (Weremczuk et al. 2016). Application of excess zinc during the agronomic fortification may lead to stress and affects normal physiology. A dose of Zn 80 μM in lettuce led to decrease in the NO^{3-} concentration, nitrate reductase (NR), glutamine synthase (GS), aspartate aminotransferase (AAT) activities, and the photorespiration processes. Lowering the Zn concentration below 80 μM increases the essential amino acids and nitrogen use efficiency in plants (Barrameda-Medina et al. 2017).

2.6 Iron (Fe) Biofortification

Iron is the major element required for human health specially for blood haemoglobin. It is also an event from the studies that the bioavailability of heme

Table 15.4 Iron content in leafy vegetables along with some other important vegetables

Sr. no.	Crop name	Iron content
Leafy vegetable crops (value in mg/100 g and fresh weight basis)		
1	*Amaranthus* (*Amaranthus* sp.)	25.50
2	*Basella* (*Basella alba*)	10.50
3	Celery (*Apium graveolens*)	6.30
4	Chinese cabbage (*Brassica chinensis*)	0.60
5	Chow chow leaves (*Sechium edule*)	0.60
6	Colocasia leaves (*Colocasia esculenta*)	0.90
7	Cowpea leaves (*Vigna unguiculata*)	20.10
8	Drumstick leaves (*Moringa oleifera*)	7.00
9	Fenugreek leaves (*Trigonella foenum-graecum*)	16.50
10	Kale (*Brassica oleracea* var. *acephala*)	1.60
11	Lettuce (*Lactuca sativa*)	2.40
12	Palak (*Beta vulgaris* var. *bengalensis*)	16.20
13	Pumpkin leaves (*Cucurbita moschata*)	2.10
14	Spinach (*Spinacia oleracea*)	15.50
15	Water spinach (*Ipomoea aquatica*)	3.10
Other vegetable crops (value in mg/g dry weight basis)		
16	Peas, dried	50
17	Cassava root	5
18	Sweet potato	6
19	Irish potato	3
20	Cabbage, broccoli	17
21	Tomatoes	5

iron (from animal sources) is more than the non-heme iron (plant sources); thus, the vegetarian requires approximately 1.8 times more iron than the non-vegetarian population. Staple foods like rice, wheat, and pulses are low in iron content, while the underutilized leafy vegetables serve as the best source of iron along with antioxidants. It can be said that these vegetables are naturally fortified with iron (Chiplonkar et al. 1999). Iron content of common leafy vegetable along with the staple food is compared in Table 15.4. Iron nutrition research has demonstrated the efficacy of biofortified iron bean and iron pearl millet in improving the nutritional status of target populations. In Rwanda, iron-depleted university women showed a significant increase in haemoglobin and total body iron after consuming biofortified beans for 4.5 months (Haas et al. 2016).

2.7 Calcium (Cr) Biofortification

Little attention has been given for calcium biofortification in vegetables because of higher quantity of calcium as compared to staple crops. The calcium biofortified carrot was developed to improve the bioavailability of calcium in the edible portions.

In the study conducted by Park et al. (2004), Ca content in carrot was increased 1.6-fold as compared to control plant. The short cation exchanger 1 (*sCAX1*), a vascular calcium/proton antiporter previously identified from *A. thaliana* (Hirschi et al. 1996) was used to achieve the target. The *sCAX1* containing carrot was normal and having equivalent yield to that of non-transformed carrots (Park et al. 2004). The utility of these carrots was further proved by Morris et al. (2008) with feeding trials of mice and humans. They however raised the question about bioavailability of the calcium and showed that the bioavailability in *sCAX1* expressing was lower as compared to normal carrot. The *sCAX1*-expressing carrots were shown to be a better source of dietary calcium because the total amount of calcium absorbed per g of carrot was significantly higher for *sCAX1*-expressing carrots than for control carrots (Morris et al. 2008). Another approach for introducing variation in *CAX1* was achieved through targeting induced local lesions in genome (TILLING) to generate plants efficient in calcium metabolism. In *Brassica rapa* subsp. *trilocularis*, Navarro-León et al. (2018) used the TILLING approach to induce mutations in the *BraA.cax1* to generate the allelic variation in the said gene. Three allelic variations were achieved at the said location, i.e. *BraA.cax1a-4*, *BraA.cax1a-7*, and*BraA.cax1a-12*. All mutants accumulated more Ca and Mg in leaves under control and high Ca doses and accumulated more Fe regardless the Ca dose. *BraA.cax1a-4* and *BraA.cax1a-7* mutants presented lower total Chl, an altered photosynthesis performance, and higher ROS levels. *BraA.cax1a-12* mutant grew better under high Ca conditions. The *BraA.cax1a-12* mutant present a good candidate for biofortification as this mutant was able to accumulate high Ca, Mg, and Fe in the leaves together with 80% leaf yield advantage over control (Navarro-León et al. 2018).

2.8 Silicon (Si) Biofortification

Regarding mineral components, in addition to iodide, calcium, and selenium, silicon is also considered a microelement important for health. Silicon is widely found in plant-based foods, drinking water, and some alcoholic beverages, notably beer (Jugdaohsingh et al. 2002; Powell et al. 2005), although its absorption depends on the food source (Sripanyakorn et al. 2009) and its chemical form. This mineral has prominent role in bone mineralization, increasing the bone density, and in general bone health (Jugdaohsingh 2007). Based on the ability of Si accumulation the crops can be grouped in three classes viz. species of Poaceae, Equisetaceae, and Cyperaceae show high Si accumulation (4% Si on dry weight); the Cucurbitales, Urticales, and Commelinaceae show intermediate Si accumulation (2–4% Si), while most other species demonstrate little accumulation. Its absorption in the intestinal tract is related to the food source. As an example, it is well absorbed from alcohol-free beer (64% of dose) and green beans (44%); in contrast, it is poorly absorbed (4%) from bananas (Sripanyakorn et al. 2009).

Achieving the Si biofortified crop through common farming is difficult as compared to the floating system (soil less) (Ferrarese et al. 2012). Using the floating system of cultivation, D'Imperio et al. (2016) produced leafy vegetables (tatsoi,

mizuna, purslane, basil, Swiss chard, and chicory) with improved Si content and available as fresh vegetable, though it was found that the accumulation was species dependent. The Si became bioaccessible in all species considered in a range from 23% (basil) to 64% (chicory). In another study, Si biofortification was achieved in purslane and Swiss Chard. The obtained biofortified vegetable was able to improve the expression of osteoblast markers (D'Imperio et al. 2017), i.e. has role in the bone mineralization. The application of Si to the nutrient solution in the range of 50–100 mg L^{-1} allows biofortification of leafy vegetables (D'Imperio et al. 2016).

Beans are high-value nutrient vegetables and offer better absorptions of Si in the intestinal tract as discussed above. To biofortify beans with Si, a soil less system approach was used. The Si concentration was increased up to three times in biofortified beans as compared with unbiofortified beans. The Si was higher even after cooking irrespective of cooking methods, in the biofortified as compared with unbiofortified beans. Si bioaccessibility in cooked pods was more than tripled as a result of biofortification, while the process did not affect the visual quality of the product (Montesano et al. 2016).

2.9 Chromium Biofortification

Chromium (Cr) is an essential trace element for human nutrition (Kimura 1996). There are two forms of chromium, i.e. trivalent (Cr^{3+}) and hexavalent (Cr^{6+}), of which the Cr^{3+} is beneficial for health, while Cr^{6+} is highly toxic. It is well studied that Cr^{3+} has important role in improving the functionality of insulin signalling, thus important for managing type 2 diabetes (Jeejeebhoy et al. 1977; Hua et al. 2012). An estimated safe and adequate quantity for daily intake of Cr^{3+} in adults ranging from 50 to 200 μg (Kimura 1996) was established by the US Food and Nutrition Board of the US Academy of Sciences. In order to biofortify chromium, fenugreek plant was selected (Priyadarshini and Brar 2020) which has also well-known medicinal role as anti-diabetic food (Eidi et al. 2007; Puri et al. 2011; Kumar et al. 2015; Gaddam et al. 2015). Thus, biofortifying fenugreek with Cr^{3+} will synergistically tackle this global epidemic. Cr biofortification of fenugreek with soil application of tannery sludge and tannery wastewater is limited by supply from soil to root and then to aerial parts (Sinha et al. 2007). Priyadarshini and Brar (2020) used chromium picolinate to treat seed and observe the accumulation of Cr in the seed flour. The seeds were treated at two doses, 0.02 g (T_1) and 0.04 g (T_2) chromium picolinate solution on first day and 0.01 g (T_1) and 0.02 g (T_2) chromium picolinate solution on second day, respectively. The seeds were dried, and flour was tested for Cr. The treated plants accumulated 163 μg Cr/g (T_1) and 236 μg Cr/g (T_2) in the flour, while the control has 1.07 μg Cr/g in the flour (Priyadarshini and Brar 2020).

2.10 Betacyanin Biofortification

Betacyanins are strong natural antioxidants, and to fortify spinach with this compound, a hydroponic experiment was carried out with three candidates which are dopamine, calcium, and sucrose. The hydroponically grown spinach was tested against these three candidates, and response towards accumulation of betacyanin was observed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and antioxidant activity analyses showed that sucrose was most successful in biofortifying spinach with betacyanin. Through reverse transcription polymerase chain reaction (RT PCR), it was identified that sucrose was able to induce the expression of betacyanin-related several genes (Watanabe et al. 2018).

3 Tapping the Richness of Vegetable Diversity in India

3.1 β-Carotene

The abundant provitamin A carotenoids are present in vegetables such as broccoli (*Brassica oleracea*), spinach (*Spinacia oleracea*), carrot (*Daucus carota*), squash (*Cucurbita maxima*), sweet potato (*Ipomoea batatas*), and pumpkin (*Cucurbita maxima*) (Jiang et al. 2017). In cauliflower, negligible β-carotene was available in commonly grown cauliflower. However, recently an orange cauliflower with β-carotene was released for cultivation in India (Kalia et al. 2018). The cause of this change attributed to a spontaneous mutation in the otherwise unpigmented tissue. The study suggests that this orange mutation (*Or*) was semi-dominant in expression, i.e. the hybrids have a bright orange colour with normal size of curd, while the homozygous *Or* produces smaller curd due to certain pleotropic effects. The molecular studies suggest that *Or* encodes a plastid-associated protein containing a DnaJCys-rich domain. The *Or* gene mutation is due to the insertion of a long terminal repeat retrotransposon in the *Or* allele. *Or* appears to be plant specific and is highly conserved among divergent plant species (Lu et al. 2006). Based on this mutant, a new variety of orange cauliflower was developed through pure line selection at ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi, containing an amount of 8–20 ppm β-carotene. The same has been released for cultivation in the year 2015 for cultivation in NCT, Delhi region (Kalia et al. 2018). ICAR-IARI has also released a carrot variety Pusa Rudhira with total carotenoid of 7.60 mg/100 g, β-carotene 4.92 mg/100 g, and lycopene 6.70 mg/100 g of root.

In case of potato, a variety, namely, *Bhusona*, has been released for cultivation which is also based on the pure line selection method of breeding. This variety has high β-carotene content as compared to the existing potato cultivars. The β-carotene content in *Bhusona* ranges from 14 mg to 100 g, while the popular cultivated varieties have 23 mg/100 g β-carotene (Yadava et al. 2017). This variety was developed for Odisha region by ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala, during the year 2017. Similarly, in sweet potato, the variety Sree Kanaka was released for cultivation having 10–14 mg/100 FW carotene

content. Another sweet potato variety rich in Sree Vardhini is having 1200 IU carotene/100 g. Sree Bhadra is a sweet potato variety with pink skin and coloured flesh with the carotene content 972 IU/100 g. Sree Rathna a variety of sweet potato has been released for cultivation having purple skin with orange colour flesh. This variety has very high content of carotene equivalent to 3500 IU/100 g. These varieties are released for cultivation from ICAR-CTCRI, Thiruvananthapuram, Kerala.

3.2 Anthocyanin

A variety of sweet potato named *Bhu Krishna* has been released for cultivation in the Odisha region of the country. This variety has very high amount of anthocyanin as compared to the negligible amount in the normal cultivars. This has an anthocyanin content of 90 mg/100 g of fresh weight (Yadava et al. 2017). ICAR-Central Potato Research Institute, Shimla, has developed and released first-ever purple-coloured indigenous specialty potato variety Kufri Neelkanth, a new table purpose medium maturing specialty potato cultivar released for North Indian plains. It is rich in antioxidants (anthocyanins >100 μg/100 g fresh wt. and carotenoids~200 μg/100 g fresh wt.). A black carrot variety Kashi Krishna (Fig. 15.2) has been released for cultivation from ICAR-Indian Institute of Vegetable Research (ICAR-IIVR), Varanasi. This variety is rich source of anthocyanin (285 mg/100 g FW carrot), phenolics, and antioxidants. ICAR-IARI also released a black carrot variety Pusa Asita with high anthocyanin content of 520 mg/100 g (Singh et al. 2019). Kashi Lohit, a radish variety (Fig. 15.3) with attractive red colour root and rich source of antioxidants specially anthocyanin 80–100% higher than white radish, has been released for cultivation from ICAR-IIVR, Varanasi. Other varieties in radish from ICAR-IARI with anthocyanin and antioxidants released for cultivation are Pusa Jamuni and Pusa Gulabi (Singh et al. 2019). In okra (*Abelmoschus esculentus* L.), ICAR-IIVR has developed Kashi Lalima, a red-/purple-coloured variety which has

Fig. 15.2 Roots of black carrot variety Kashi Krishna

Fig. 15.3 Roots of radish variety Kashi Lohit

Fig. 15.4 Plants and fruits of red okra variety Kashi Lalima

Fig. 15.5 The plant, pods, flower, and field view of the French bean variety Kashi Baingni

been first ever released for cultivation, and it has high anthocyanin content as compared to green-coloured okra (Fig. 15.4). This variety became popular among farmer within a short span of time and highly liked by the consumers. During the year 2020, a variety Kashi Baingani (Fig. 15.5) in nutritionally very important crop French bean has been released for cultivation by ICAR-IIVR. The purple-coloured French bean variety has high antioxidants and rich in anthocyanin.

3.3 Betalain

Betalain is nitrogenous pigments restricted to the members of order *Caryophyllales.* Betalains are further classified in two groups, i.e. betacyanins (purple) and betaxanthins (yellow). These pigments are water soluble and are stored in the vacuoles of the cell (Robinson 1999). Various bioactive properties were reported to be present in this pigment, not only to protect the plants but also to the consumer of plant products. Studies suggest anti-cancerous properties (Khan et al. 2012), reduction in induced tumour (Lechner et al. 2010), anti-inflammatory (Reyes-Izquierdo et al. 2014), health promoting, and reduced oxidative stress (Guerrero-Rubio et al. 2019). Indian spinach (*Basella alba* L.) and Amaranth (*Amaranthus tricolor* L.) are two leafy vegetables with high betalain content in leaves, stem, and fruits. These two vegetables occupy significant kitchen garden spaces in the country. Several varieties of Amaranth had been released in the past; however, only few varieties are available in the Indian spinach. Recently during 2019, three varieties of Indian spinach, namely, Kashi Poi1, Kashi Poi 2 and Kashi Poi3, were released for

Kashi Poi-1 Kashi Poi-2 Kashi Poi-3

Fig. 15.6 Showing different varieties of *Basella rubra* released for cultivation

Fig. 15.7 Plants of *Amaranthus* cultivar Pusa Lal Chaulai

cultivation from ICAR-IIVR, Varanasi (Fig. 15.6). The popular amaranth variety was Pusa Lal Chaulai (Fig. 15.7) having purple leaves with high yield potential.

3.4 Mineral and Vitamin-Rich Moringa

Moringa is considered as the native crop of India with centre of origin in the Himalayan tracts. The wild type of moringa is generally perennial in nature, whereas the improved moringa cultivars are annual. The moringa is used for almost all the parts; however, most readily consumed are its fruits, leaves, and flowers. The leaves of moringa are consumed in different forms like leaf powder and freshly cooked saag.

4 Methods of Biofortification

Biofortification can be achieved through the following three strategies as discussed below.

4.1 Agronomic Biofortification

The agronomic biofortification can be achieved by application of fertilizers to increase the micronutrients in edible parts (Prasad et al. 2015). Most suitable micronutrients for agronomic biofortification are zinc (foliar applications of ZnSO4), iodine (soil application of iodide or iodate), and selenium (as selenate). Foliar application is the quick and easy method of nutrient application to fortification of micronutrients (Fe, Zn, Cu, etc.) in plants. Agronomical approaches, viz. seed treatments, foliar application, and organic manures could be used for increasing the nutritional values in various vegetable crops (Fig. 15.8), are comparatively less expensive and quick as compared to any other methods of improvement. However, these techniques are useful for elevating mineral contents in various vegetables. The contents of various phytochemicals like terpenes, chlorophylls, polyphenols, and organosulphur compounds cannot be fortified by using agronomical techniques.

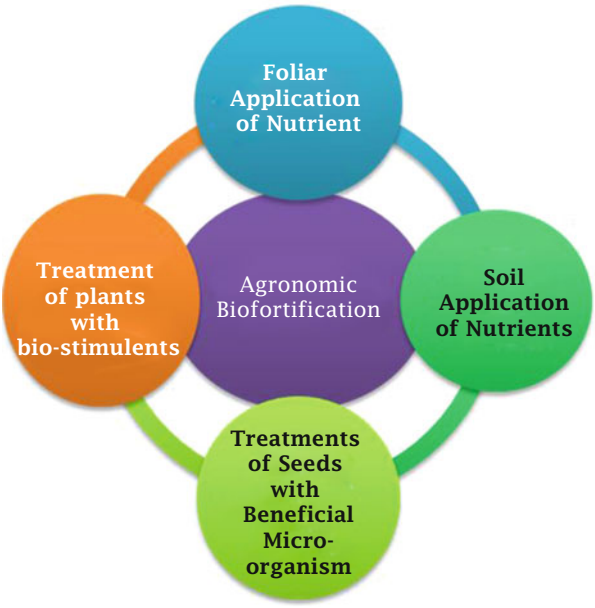

Fig. 15.8 Strategy of biofortification using agronomic means

4.2 Conventional Plant Breeding

The potential to increase the micronutrient density of staple foods by conventional breeding requires adequate genetic variation in concentrations of β-carotene, other functional carotenoids, iron, zinc, and other minerals which exist among the cultivars, making selection of nutritionally appropriate breeding materials possible. It starts with germplasm screening for the trait, inheritance studies, physiological, or bioavailability studies and finishing with product development in the form of new biofortified varieties. Research on biofortification of cow pea was initiated, and two early maturing high iron- and zinc-fortified varieties, namely, Pant Lobia-1 (82 ppm Fe and 40 ppm Zn), Pant Lobia-2 (100 ppm Fe and 37 ppm Zn), have been developed by conventional plant breeding and released in 2008 and 2010 (Gomathi et al. 2017). Popular conventional breeding methods like selection, introduction, and hybridization have been exploited for developing nutraceuticals in vegetables as well as tuber crops (Fig. 15.9). Several sources of high nutraceuticals have been identified and transferred in popular cultivars through traditional breeding methods. This method uses intrinsic properties of crop; however, it may take comparatively very long time for developing new variety, and the success of the breeding programme depends upon the available variability. In India, many varieties were developed in various vegetables and tuber crops. The Indian Agriculture Research Institute (IARI) has strengthened the work on development of biofortified vegetables specifically on temperate vegetables. Several donor parents have been identified in different

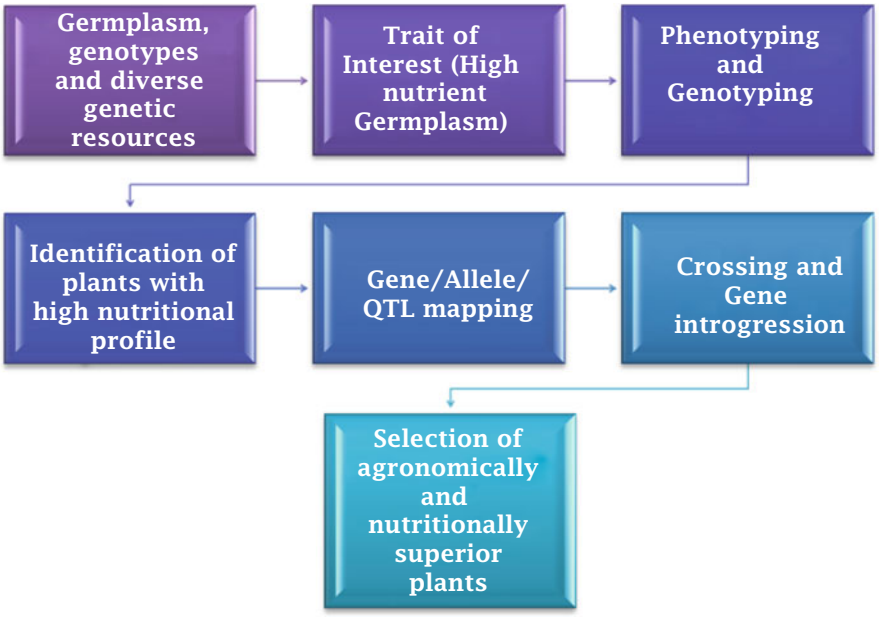

Fig. 15.9 Strategy for development of biofortified varieties through breeding

vegetables having high nutraceutical values. In cauliflower, Pusa Beta Kesari 1 has been released in 2015 as first biofortified variety through pure line selection containing high β-carotene (8.0–10.0 ppm) as compared to negligible β-carotene content in most of the popular varieties of cauliflower. Some research work on developing nutraceutical varieties has been initiated by the Indian Council of Agricultural Research (ICAR), New Delhi. In carrot, Pusa Rudhira has been released which is nutritionally rich as compared to other carrot varieties. The variety was tested to have higher levels of carotenoid (7.41 mg) and phenols (45.15 mg 100 g^{-1}). In radish, the pink- and purple-fleshed radish varieties were released by the Indian Agriculture Research Institute (IARI). Pusa Gulabi is the first pink-fleshed radish variety released in 2013 which is high in total carotenoids, anthocyanin, and optimum in ascorbic acid content, whereas Pusa Jamuni is the first purple-fleshed nutritionally rich variety high in anthocyanin and ascorbic acid content.

4.3 Biotechnological Interventions

Due to lack of sufficient variation among the genotypes for the desired character/trait within the species, or when the crop itself is not suitable for conventional plant breeding (lack of sexuality) then genetic engineering offers a valid alternative for increasing the concentration and bioavailability of micronutrients in the edible crop tissues (Fig. 15.10). Genetic engineering enables vegetable breeders to incorporate desired transgenes into elite cultivars, thereby improving their value considerably. It further offers unique opportunities for improving nutritional quality and bringing other health benefits. Many vegetable crops have been genetically modified to improve traits such as higher nutritional status or better flavour, to reduce bitterness, slow ripening, higher nutritional status, seedless fruit, increased sweetness, and to reduce antinutritional factors. Transgenic carrots have been reported to express increased levels of the plant Ca transporter SCAX1 (Lee et al. 2003). In crops where the target nutrient does not naturally exist at the required levels in the tens

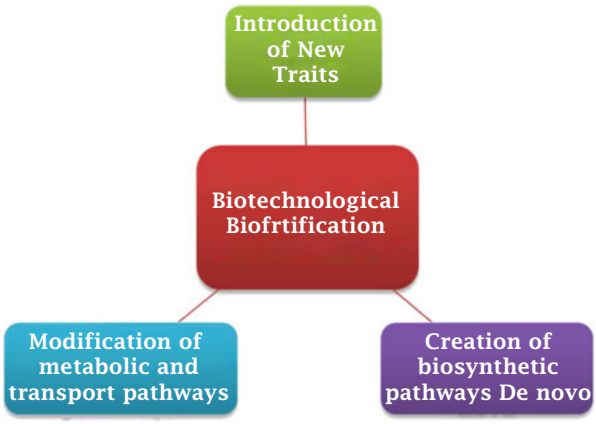

Fig. 15.10 Strategies of biotechnological interventions in biofortifications

of thousands of varieties in germplasm banks, transgenic plant breeding is a promising approach to produce biofortified crops with the desired nutrient and agronomic traits. For example, transgenic iron and zinc rice have been developed and tested in confined field trials that can provide 30% of the EAR for both nutrients (Trijatmiko et al. 2016). Golden rice, which contains beta carotene, can provide more than 50% of the EAR for vitamin A. Many vegetable crops have been genetically modified through various transgenic techniques for several nutritional traits. In sweet potato, to increase the levels of carotenoids, transgenic sweet potato plants overexpressing IbOr-Inscan under the control of the cauliflower mosaic virus (CaMV) 35 S promoter in an anthocyanin-rich purple-fleshed cultivar (referred to as IbOr plants) was developed. IbOr plants exhibited increased carotenoid levels (up to sevenfold) in their storage roots compared to wild-type (WT) plants. In tomato, the strategy adopted involved pathway extension beyond β-carotene through the expression of the β-carotene hydroxylase (CrtZ) and oxygenase (CrtW) from *Brevundimonas* sp. in tomato fruit, followed by β-carotene enhancement through the introgression of a lycopene β-cyclase (β-Cyc) allele from a *Solanum galapagense* background.

5 Conclusions

Even after two decades of research on biofortification, its impact is sporadically visible. Previous efforts mainly focusing on using transgenics had faced strong regulatory hurdles and protest even before getting released for commercial cultivation. In this situation, the most viable option remains as conventional plant breeding and agronomic biofortification. Significant achievement has been made in utilizing the existing natural variation and development of commercial varieties through conventional plant breeding in cereal crops as well as vegetable crops. Also, several successful examples are available for agronomic biofortification; however, its large-scale production remains a question mark. Vegetable offers an array of diverse food with mineral nutrient and nutraceuticals on regular basis throughout the year. Now is the time for policy-makers and thinker to streamline the vegetable consumption through awareness and policy changes so that it can reach the needy people who don't have access to supplements and pills. Vegetable biofortification should be seen as the sunrise sectors for farmers and consumers in benefit sharing. The farmers will get the premium price for their fortified vegetable, and consumer will get the benefit of improved nutritional status. The future aspect of vegetable biofortification research should be focused on combining the high mineral and vitamins along with nutraceutical component. More and more use of marker assisted breeding for rapid gains and accurate results. Improved cultivars of underutilized vegetable crops should be promoted in the areas where such communities' preferences exist. Vegetable biofortification along with other biofortified crops can help in achieving the sustainable development goal targets. However, it will be efficient to explore a holistic development plan involving crop breeder (agriculturist), health expert, and other private players for PPP mode collaboration.

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Biofortification of Cassava: Recent Progress and Challenges Facing the Future

16

Richard T. Sayre

Abstract

Cassava (*Manihot esculenta*) ranks as the fifth most important crop in the world that is consumed directly by humans. Cassava is a tropical crop of South American origins but is most important as a staple crop in the diet of Sub-Saharan Africans where it is valued for the food security it provides as well as by consumer preference. While the starchy root of cassava is a valuable source of calories, it does not provide sufficient protein, iron, zinc, or β-carotene in a typical sized meal to meet minimum daily nutritional requirements. Furthermore, cassava contains potentially toxic levels of cyanogenic glycosides and is impacted negatively by a short shelf-life following harvesting that limits the amount of cassava that can be harvested and processed by subsistence farmers for direct consumption or marketing. To address these challenges, multiple international and national breeding and genetic engineering programs are focusing on developing farmer-preferred varieties that meet the minimum daily requirement for complete human protein and micronutrient needs in a cassava meal. Significantly, biofortification of cassava has been demonstrated to be economically the most efficient strategy to meet the nutritional needs of consumers who largely subsist on a cassava-based diet. In the following review, we discuss the substantial progress that has been made in the biofortification of cassava and address the challenges facing the future.

Keywords

Cassava · *Manihot esculenta* · β-Carotene · Biofortification · Genetic engineering · Micronutrient malnutrition · Protein-energy malnutrition · Molecular-assisted breeding · Vitamin A

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Abbreviations

AGPase	ADP glucopyrophosphorylase
CIAT	Centro International Agricultura Technologia
FAO	Food and Agricultural Organization
gdw	Grams dry weight
GS	Genomic selection
IITA	International Institute for Tropical Agriculture
MDR	Minimum daily requirement
MNM	Micronutrient malnutrition,
PEM	Protein energy malnutrition
SSA	Sub-Saharan Africa
UNICEF	United Nations International Children's Emergency Fund
WHO	World Health Organization

1 Introduction

The global problems of food undernourishment, malnutrition, and food insecurity are complex and interrelated in terms of causality. Factors including the inherent nutritional quality of food, and stochastic events including intermittent access to irrigation water, fertilizers, pesticides, and herbicides all impact food security and nourishment. In addition, the cost of food, barriers to distribution, food preparation practices, wastage, and spoilage can profoundly impact global nutrition. Today, however, in many regions of the world access to sufficient nutritious foods is being challenged. According to a recent United Nations report entitled *State of Food Security and Nutrition in the World,* jointly published by the FAO, UNICEF, and WHO, it was estimated that in 2019 nearly 690 million people or 9% the global population was chronically undernourished. Significantly, this number represents an increase of nearly 60 million persons since 2014 (FAO 2020). In addition, the number of people impacted by moderate or severe food insecurity, defined as not having food for at least 1 day, is estimated to be two billion people. Global estimates of the population suffering from protein and micronutrient malnutrition mirror levels of global food insecurity (Ramakrishnan et al. 2009). According to recent estimates (2019), 21.3% (144.0 million) of all children under the age of 5 years are malnourished with 6.9% (47.0 million) of children being severely malnourished. Chronic malnutrition can lead to permanent stunting (stunting is defined as a stature height two standard deviations from the median for a given age) and irreversible reduction in IQ, resulting in substantial loss in income potential over one's life (Black et al. 2008; Fiedor and Burda 2014). Even greater is the loss of life estimated to be one million children under the age of 5 due to malnutrition.

Significantly, not all regions of the world experience similar levels of food insecurity and malnutrition. Undernourishment and malnutrition are most prevalent

in Sub-Saharan Africa (SSA) where in 2019, 19.1% of the population or more than 250 million people were undernourished, an increase of 17.6% since 2014. This level of undernourishment is more than twice the world average (8.9%) and the highest of any region in the world. Furthermore, UN projections indicate that undernourishment in SSA is likely to increase to 26% of the total regional population by 2030. Clearly, the challenge of providing adequate nutritious food is great and must be engaged at a variety of levels including addressing income disparity, application of advanced and digital agricultural practices, improved food distribution and affordable marketing. At the consumer level, there is a need for further education and access to adequate diverse sources of food to provide a balanced and affordable diet and/or consistent supplies of supplemental micronutrients to address dietary deficiencies.

Until the 1980s, most of the focus on addressing chronic undernourishment or malnutrition in the world focused on protein energy malnutrition (PEM) while initiatives to address micronutrient malnutrition (MNM) were fewer and more recently emerging. It has been estimated, however, that MNM may contribute to 7.3% of the total global disease burden in the world, particularly iron and vitamin A deficiency, which accounts for or contributes to nearly one million deaths each per year. In terms of disability-adjusted life-years lost (DALY; defined as the number of years of life lost and the number of years lived with temporary or permanent disability due to a given health problem), iron deficiency, and vitamin A deficiency account for 25 million and 18 million DALYs lost globally each year. Zinc deficiency also remains a significant health impact in various regions of the world.

To address the challenges of food insecurity, PEM, and MNM, a regional crop-specific approach must be taken, taking into the account what are the dominant staple crops consumed in a given region, the diversity and nutritional composition of foods that are consumed on a daily basis, and food costs and food availability (Sautter et al. 2006; Sayre 2011; Sayre et al. 2011). In SSA, cassava is one major staple crop providing a relatively secure source of calories while requiring limited agronomic inputs. In addition, cassava is drought tolerant and its starchy roots can be banked in the soil for long periods of time providing additional food security. The presence of potentially toxic levels of cyanogenic glycosides in cassava also provides some level of food security against theft and herbivory (Ernesto et al. 2002; Gleadow and Møller 2014; McMahon et al. 1995; Sayre 2011).

In 2008, over 118 million tons of cassava were harvested in SSA. Globally, cassava ranks as the fifth most important source of calories that are directly consumed from plants. A typical sized (500 g) cassava meal provides sufficient calories in the diet but is substantially lacking in protein, iron, β-carotene or provitamin A, and zinc (Table 16.1) (Montagnac et al. 2009). The protein content of cassava storage roots is among the lowest of all major crops. For an adult, the daily recommended caloric and protein intake is 2300 kCal and 69 g, respectively. Cassava roots typically have between 0.7 and 3% protein by dry weight. Thus, a typical sized cassava meal of 500 g would provide 77% of the daily caloric intake but only 8% of the daily protein requirement (Table 16.1). The low protein content in cassava-based foods is also impacted by the type of food processing and preparation

Table 16.1 Nutritional qualities of cassava foods in a 500 g meal (FAO). Assumed a 12:1 β-carotene to retinal conversion ratio

Minimum daily requirement (MDR)	Energy 1700–2400 (kCal)	Protein 50–80 (g)	Iron 18 (mg)	Zinc 12 (mg)	Vitamin A (β-carotene) 11 (mg)
Fresh cassava (% MDR)	745 (36)	6 (9)	2 (11)	2 (17)	1 (9)
Dried chips (% MDR)	1775 (87)	10.5 (16)	4 (22)	4 (34)	2 (18)
Flour (% MDR)	1710 (83)	7.5 (12)	4 (22)	3 (25)	0 (0)
Boiled (% MDR)	740 (36)	5.5 (9)	2 (11)	2 (17)	1 (9)
Roasted (% MDR)	1360 (66)	10 (15)	2.5 (13)	3 (25)	1 (9)

used to remove cyanogens (Cardoso et al. 2005). As a result, subsistence on a cassava-based diet crop can result in protein energy malnutrition (PEM) unless there are additional sources of protein in the diet. Micronutrient malnutrition associated with subsistence on a cassava-based diet is also a concern. A typical adult cassava meal provides between 10 and 20% of the MDR for iron, zinc, and provitamin A or β-carotene (Table 16.1) (Charles et al. 2005; Kimura et al. 2007). It is estimated that over one billion people worldwide are affected by iron-deficient anemia. In Sub-Saharan Africa alone, 50% of the population is thought to suffer from iron deficiency. In addition, as many as 32% of children under the age of 5 years may be disabled due to iron deficiency and as many as 16% of children die as a result of the effects of iron deficiency in SSA. In Nigeria, SSA's most populous country and largest consumer of cassava, 75% of preschool children and 67% of pregnant women are anemic, and 20% of children under 5 years of age have zinc deficiency, causing increased risk to stunting suppression of the immune system and reduction in cognitive development. In addition, 83% of the children in Nigeria between the ages of 2–5 years of age exhibit vitamin A deficiency.

In addition to poor nutritional composition, cassava roots have a short shelf life (1–3 days) following harvesting from the plant due to rapid postharvest deterioration (PPD). This rapid degradation of cassava roots limits the area that can be harvested by subsistence farmers to that area or amount of cassava roots that can be processed in a few days into stable food products. Thus, the problem of PPD limits the application of large-scale farming practices or long-distance transport to markets to generate income. Additionally, cassava is particularly susceptible to several vector-borne viral diseases that can devastate crop yields.

To identify the most effective means to address PEM and MNM associated with a cassava-based diet, it is necessary to estimate the effectiveness and relative costs of competing intervention strategies. One approach to estimate the cost–benefit analyses of competing technologies for cassava biofortification is to calculate the economic value of disability-adjusted life-years (DALY is defined as the loss of the

equivalent of 1 year of full health; DALYs for a disease or health condition are the sum of the years of life lost due to premature mortality and the years lived with a disability due to prevalent cases of the disease or health condition in a population) saved by the intervening technology as functions of (1) the level of biofortification, (2) the proportion of the population adopting the modified food product, (3) the age-dependent impact of the intervention, and (4) the cost for developing the intervention. In 2011, it was estimated that for biofortified cassava varieties providing 100% of their micronutrient dietary needs taken in a meal, which provided 25% of the daily caloric needs and which was adopted by 24% of all consumers that the biofortified cassava would reduce annual DALYs attributed to iron and vitamin A malnutrition in Nigeria by 6% for each micronutrient (Nguema et al. 2011; La Frano et al. 2013).

If iron and provitamin A biofortified cassava were universally adopted and presented as stacked traits in a single variety, the potential reduction in annual DALYs lost due to combined iron and vitamin A deficiency would be reduced by nearly 50% assuming no synergisms between iron and vitamin A micronutrient deficiencies. The estimated cost per combined iron and vitamin A DALY saved per year would be US$4. In comparison, the WHO estimated that the cost per DALY saved by vitamin A micronutrient supplementation would be $52/year in 2010. Finally, by assessing a value of $1000 per DALY saved the estimated value of combined iron and vitamin A biofortified cassava food products meeting 100% of the MDR in a 500 g meal and consumed at an adoption rate of 24% in Nigeria would be US $234 million/year. Clearly, the costs and benefits associated with direct crop biofortification are substantial even at low adoption rates and are substantially less costly than addressing micronutrient malnutrition by non-food supplementation.

Due to its importance as a staple crop in SSA, and its major role in the diet for people living in many countries in South America and Southeast Asia, a number of NGOs, government and international agricultural centers have developed cassava crop improvement programs to address food security and nourishment issues. These research centers include, among others, the Center for International Tropical Agriculture (CIAT) in Colombia, The International Institute for tropical Agriculture (IITA) in Nigeria, the National Root Crops Research Institute in Nigeria, the Kenyan Agricultural Research Institute (KARI), EMBRAPA in Brazil, and ICARDA in India. These agricultural research centers have emphasized traditional and advanced breeding technologies for the development of higher-yielding, disease-resistant, and in some cases biofortified cassava (Ceballos et al. 2016, 2020; de Freitas et al. 2018; Dawson et al. 2019). Targeted improvements are often developed in consultation with regional farmers and prioritized to address their needs and preferences for crop improvements. The challenges for the cassava breeder are somewhat unique among the world's major food crops, however.

Cassava is monoecious, and seed production can often be low for some varieties. Furthermore, flowering time may be asynchronous between varieties. But perhaps most importantly cassava is almost universally propagated clonally by stem cuttings by farmers. Thus, a seed-based industry for cassava crop improvement has not materialized. As a result, commercial economic incentives to support crop breeding

and a seed improvement and production industry have been lacking. However, the clonal propagation of cassava does lend itself well to genetic engineering strategies for crop improvement since the requirement for extensive and long-term backcrossing to develop inbred hybrids is less critical with genetically modified clones to ensure consistent generation-to-generation crop performance.

In the following sections, we review the impact of traditional and advanced breeding technologies and genetic engineering approaches on addressing the challenges of cassava biofortification. We will also address the challenges unique to cassava including the presence of antinutrients or cyanogenic glycosides and the issue of short root shelf life that limits yields and income generated from cassava harvests due to processing constraints needed to remove toxic cyanogens (linamarin and acetone cyanohydrin) (Okafor 2004). The encouraging news is that substantial progress has been made on multiple technology fronts to improve cassava nutrition. What is evident, however, is that the financial support or interest for taking foundational research from the lab to the field has limited the application of technologies that could potentially impact the lives of many persons. This issue, that is, the failure to move basic research to field applications, is particularly concerning given the growing increase in food insecurity and undernourishment since 2014 and the projected worsening of food security in SSA through 2030.

2 Cassava Breeding and Biofortification

The development of advanced breeding programs for cassava has been challenged by its biology. Cassava is a diploid ($2n = 36$) non-inbred highly heterozygous crop that has a long breeding cycle requiring minimally 6–8 years to develop new varieties (Chavez et al. 2005). The crop is also hampered by low seed output and asynchronous flowering between varieties. It has been proposed that the development of synchronous flowering traits would substantially enhance cultivar development rates (Chiurugwi et al. 2019).

Marker-assisted selection has been successfully employed to breed for traits that have known large-effect loci such as cassava mosaic disease resistance but more complex multi-loci traits such as micronutrient enhancement are not readily amenable to marker-assisted breeding programs (Friedmann et al. 2018). Relevant to addressing the challenges of PEM and MNM are farmer preferences for improved agronomic traits in cassava. With the exception of β-carotene, which is brightly orange colored, PEM- and MNM-associated traits (iron, zinc, protein, etc.) are often called "invisible traits" since they are not readily identifiable by farmers or consumers (Ariza-nieto et al. 2006). A recent survey of farmer trait preferences in Nigeria ranked crop yield and cooking traits as the highest priorities for crop improvement. PEM (protein content) and MNM (vitamins and minerals) traits were not identified as traits of interest at all (Table 16.2).

As will be discussed below, however, there has been success in elevating β-carotene (provitamin A) levels in cassava using marker-assisted selection breeding strategies (Ilona et al. 2017). Potentially more complex heritable traits such as

Table 16.2 Cassava trait preferences of Nigerian framers adopted from Teeken et al. (2018)

Trait prioritized rankings	Frequency mentioned by respondents (%)
Yield	73
Root size	60
Early maturing	55
Dry matter content	43
Cooking quality	40
Flesh color	38
Shelf life	38
Ease of root processing	37
Cooking quality	32
Price	29
Agronomic characteristics	26
Taste	25
Resistance to pests and disease	21
Abiotic stress resistance	10
Labor requirement	5

elevated inorganic nutrient content, however, may require more advanced breeding strategies such as machine learning-assisted genomic selection (GS) tools to accelerate and advance the development of elite biofortified cassava varieties (Lima et al. 2019; Ozimati et al. 2019).

Over the last 50 years, a variety of genomic resources and tools have been applied to cassava breeding programs to develop superior cultivars including quantitative trait loci (QTL) mapping and assisted breeding, genome-wide association studies (GWAS), and genomic selection (GS) tools (Ramu et al. 2017; Wolfe et al. 2017). Implementation of assisted breeding strategies, however, is at various stages of development and implementation throughout the world (Barandica et al. 2016; Rabbi et al. 2017). GWAS and GS represent substantial computational and statistical advancements over phenotypic recurrent selection programs that dominated breeding programs until recently (Njoku et al. 2015). These advanced breeding strategies were largely made possible by the implementation of advanced high-throughput and lower-cost genomic sequencing tools. Thus, they are gaining favor in national orphan crop breeding programs that are often resource limited.

GWAS is based on advanced genomic sequencing analyses that allows for the generation of dense marker maps across the entire genome. A general requirement for GWAS is that genes associated with quantitative traits are expected to be in linkage disequilibrium with their corresponding genetic markers. GS breeding strategies involve the prediction of breeding values and selection of parents based on marker-estimated effects, enabling more cycles of selection and recombination per unit time than phenotypic recurrent selection. However, for GS approaches to be successful, the level of genetic variability and the heritability of the traits must be independently assessed within each unique breeding program.

Recently, various GS machine learning algorithms were trained and compared for their ability to select for complex traits using cassava cultivars from multiple breeding programs. In the largest cassava GS study carried out to date, Wolfe et al. (2017) assessed the performance characteristics of seven different GS models for seven different traits using cultivars obtained from three different cassava breeding programs in Nigeria and Uganda. The seven traits that were assessed included root percent dry matter, fresh root weight, root number per plot, shoot weight, harvest index per plot, severity of cassava mosaic disease, and plant vigor. For the genomic prediction programs, a total of 155,871 single-nucleotide polymorphism markers were identified and tracked. As expected, the highest predictive correlation accuracy for a trait and its associated genetic markers was shown to depend on multiple variables including the GS model used, the breeding program assessed, and the population sizes used for training the GS models. Overall, cross-validated predictive accuracies ranged as high as 0.60, indicating substantial potential for GS to accelerate cassava breeding programs. The application of GS to cassava breeding, however, is still in its infancy but promises to accelerate the development of single varieties combining diverse and complex traits. This capability will be critical to apply to PEM and MNM programs. To date, however, cassava breeding programs have been successful in addressing only a single MNM trait, provitamin A content.

2.1 Breeding for Elevated Carotenoid Levels in Cassava Roots

Recently, the National Root Crops Research Institute in Nigeria compared cassava phenotypes varying in their carotenoid content and composition using a variety of GS programs to assess genetic correlations with carotenoid phenotypes (Azevedo et al. 2016). The association between total carotenoid content and the individual carotenoids (all-trans β-carotene, violaxanthin, lutein, 15-cis β-carotene, 13-cis β-carotene, α-carotene, 9-cis β-carotene, and phytoene) were all shown to be significantly illustrative of their common metabolic origins (Aragon et al. 2018). Cross-validated correlations between the actual and estimated carotenoid values using a random forest GS program ranged from 0.62 for phytoene to 0.97 for all trans β-carotene, the most effective carotenoid substrate for the production of retinol (vitamin A).

In addition, independent GWAS analyses revealed significant carotenoid content correlated genomic regions located on multiple chromosomes and significantly an association with a locus encoding phytoene synthase (psy) (Rabbi et al. 2017). Related to this observation, Welsch et al. (2010) showed that a genetic polymorphism in the *psy* locus of yellow cassava varieties was associated with elevated carotenoid content. They also demonstrated that a polymorphic phytoene synthase had higher enzymatic activity than the enzyme from low carotenoid content cultivars, and that when the high-activity enzyme was expressed in transgenic yeast and bacteria it resulted in substantially elevated carotenoid content. These results suggested that production of phytoene in cassava may be a bottleneck in the

accumulation of carotenoids and β-carotene. Importantly, numerous studies with breeding cassava populations and genetically modified cassava having elevated β-carotene content indicate that there is a negative correlation between β-carotene levels and dry matter content. This relationship was unanticipated. In a study of three yellow and three white-fleshed African cassava cultivars mated to generate nine F1 populations, it was determined that total carotenoid content was negatively correlated with dry matter content across all temporal evaluation stages and trial locations.

More recently, it has been demonstrated that the genes involved in carotenoid production are linked to genes that could potentially reduce starch accumulation. A major locus for root carotenoid content was identified on chromosome 1 at position 24.1 Mbp. Significantly, a single locus for dry matter content was also located near the 24.1 Mbp peak for carotenoids. Genes for carotenoid (*phytoene synthase*) synthesis and sucrose synthesis (*UDP-glucose pyrophosphorylase* and *sucrose synthase*) were subsequently identified on chromosome 1 at this locus (24.1 Mbp). Significantly enhanced sucrose synthesis catalyzed by *UDP-glucose pyrophosphorylase* and *sucrose synthase* would lead to reduced starch production by channeling glucose away from starch production. However, studies on the correlation between dry matter content and β-carotene levels in South American cassava cultivars having β-carotene levels ranging from 2.4 to 15 μg/gdw demonstrated that there was a range of correlation coefficients between β-carotene and dry matter content ranging from −0.12 to 0.10 depending on the growing season. These results suggested that there was a lack of correlation between the two traits in South American cassava varieties.

Further evidence that carotenoids impact dry matter production has come from transgenic studies in which carotenoid levels were elevated in genetically engineered cassava and potato. In cassava transgenic lines having the highest carotenoid levels (~100 μg/gdw), there was a 50–60% reduction in dry matter content. Similarly, in potato plants engineered to have enhanced carotenoid accumulation, there was substantially reduced dry matter (−50%) and starch content associated with elevated sucrose levels. The molecular basis for the low starch content in high carotenoid lines came from transcriptome analyses. These studies revealed that there was reduced expression of genes involved in starch biosynthesis in high β-carotene lines including ADP-glucose pyrophosphorylase, (AGPase), the enzyme that catalyzes the rate-limiting and first-dedicated step in starch synthesis (Geigenberger 2003). These results indicate that elevated β-carotene levels can directly lead to reductions in starch accumulation at a molecular level, implying that the linkage between carotenoid synthesis genes and sucrose synthesis genes cannot solely account for the reductions in dry matter accumulation in high carotenoid cassava lines. These results demonstrate the importance of using both breeding and transgenic approaches to achieve greater understanding of the relationships between traits that are often not resolved by one genetic approach alone.

2.2 Addressing Protein Energy Malnutrition in Cassava Roots and the Central Role of Cyanogen Metabolism

As previously discussed, the protein fraction of a typical (500 g) cassava meal ranges from 3 to 15 g depending on how the food is processed to remove cyanogens. On average, a cassava meal may provide 15% of the MDR protein required for an adult. The BioCassava Plus Program had a mandate to provide complete nutrition in a single meal. To address potential protein deficiency resulting from eating a cassava-based diet, the BioCassava Plus program explored a variety of transgenic strategies to elevate root protein levels including (1) increasing root free amino acid pools for protein synthesis, (2) generating a strong nitrogen sink in roots by overexpressing storage proteins, and (3) a combination of both strategies (Stupak et al. 2006; Sayre et al. 2011).

To address the challenge of elevating root protein levels, however, we must consider the role of cyanogen metabolism in the cassava plant. The leaves and roots of cassava plants accumulate between 200 and 1300 mg CN equivalents/kg dry weight largely in the form of the cyanogenic glycoside, linamarin. As shown in Fig. 16.1, linamarin is synthesized in the leaves from the amino acid valine and transported to roots where it has two fates, metabolism to provide reduced nitrogen for assimilation into amino acids for protein synthesis or storage in the vacuole to serve as a herbivore feeding deterrent (Andersen et al. 2000; Jorgensen et al. 2005). Linamarin stored in vacuoles is stable and nontoxic until the tissue is disrupted

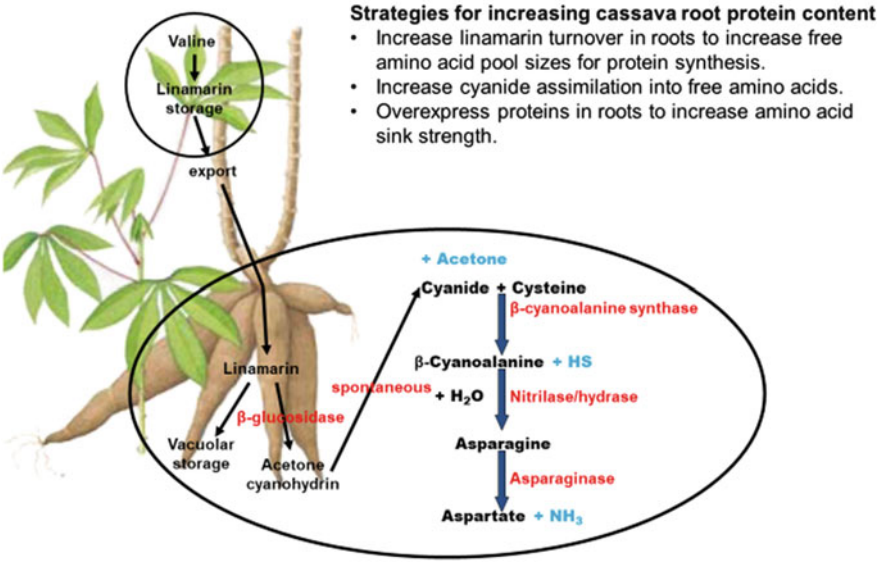

Fig. 16.1 Cyanogen synthesis, transport, and its role in protein synthesis in cassava roots. Compounds in blue font are co-products of enzymatic reactions that are not part of amino acid biosynthetic pathways

releasing the linamarin, which then interacts with the β-glucosidase, linamarase, localized in the cell walls and laticifers (Mkpong et al. 1990; McMahon et al. 1995). These disruptive events initiate hydrolysis of linamarin-producing acetone cyanohydrin. Acetone cyanohydrin can spontaneously decompose to yield cyanide and acetone at pH $>$ 5.0 or temperatures $>$35 °C, or is broken down by the enzyme hydroxynitrile lyase (HNL), which is expressed only in cassava leaves and stems and not in roots (White et al. 1998).

Several studies have demonstrated a role for cytoplasmic root linamarin in amino acid synthesis and protein accumulation. In plants, there are multiple metabolic fates for free cyanide, presumably to reduce its toxic effects on mitochondrial respiration. Two dominant cyanide assimilation pathways have been identified in plants, one catalyzed by rhodanese (cyanide: thiosulfate sulfurtransferase) leading to the production of the dead-end metabolic product thiocyanate and the other leading to the production of asparagine catalyzed by β-cyanoalanine synthase (CAS) and nitrilase (Hatzfeld and Saito 2000; Maruyama et al. 2001; Cardoso et al. 2004; Lai et al. 2009; Machingura et al. 2016). CAS catalyzes the reaction between cyanide and cysteine to form β-cyanoalanine and hydrogen sulfide, whereas nitrilase hydrates β-cyanoalanine to produce the amino acid asparagine that can serve as a reduced nitrogen donor (NH_3) for the synthesis of other amino acids (Ernesto et al. 2000; Hatzfeld et al. 2000). To determine if it was possible that cyanide could be partitioned into either the rhodanese or amino acid synthesis pathway, the relative activities of the cyanide assimilatory enzymes were assessed in different cassava organs. In addition, transgenic plants were generated that overexpressed CAS and nitrilase in roots and their effects on root-free amino acid pool sizes, protein content, and linamarin steady state pool sizes were assessed. Foremost, it was demonstrated that roots have substantially elevated CAS (2.5X) and nitrilase activity compared to leaves and virtually no rhodanese activity, suggesting the potential for active cyanide assimilation into amino acids in roots. In addition, overexpression of CAS and nitrilase was shown to lead to elevated free amino acid pool sizes (30% greater than wild type) and root protein content (10% greater than wild type), indicating that these enzymes facilitate cyanide assimilation. Further evidence that linamarin serves as a source of reduced nitrogen for amino acid and protein synthesis came from studies where linamarin turnover was increased by targeting linamarase to the vacuole. In transgenic plants in which linamarase was targeted to the vacuole (VL plants), there was a 2.5-fold increase in root total free amino acids relative to wild-type plants and a 44% reduction in root linamarin levels. There was not, however, an increase in root protein levels in VL plants. When the storage protein sporazein was co-expressed with VL to create an amino acid sink, there was both 2.4-fold increase in free amino acids, a 2.0 X increase in root protein levels, and a 45% reduction in root linamarin levels. Similar results were observed in transgenic plants overexpressing a root targeted protein hydroxynitrile lyase (HNL), which catalyzes the conversion of acetone cyanohydrin to cyanide and acetone and importantly has a balanced amino acid composition (Tabe and Higgins 1998; Narayanan et al. 2011b; Zidenga et al. 2017). As indicated earlier, HNL is only expressed in leaves of cassava and not in roots. Transgenic plants overexpressing HNL in roots

had a three-fold increase in root protein levels and an 80% reduction in root linamarin levels, indicating that an increase in amino acid sink strength could drive reductions in linamarin content consistent with its use as a substrate for amino acid synthesis. Overall, these results indicate the metabolic engineering strategies based on enhancing linamarin turnover, root cyanide assimilation into free amino acids, and enhanced protein sink strength or production in roots all lead to substantially increased total root protein content and substantially reduced linamarin content. Furthermore, root processing time to eliminate residual acetone cyanohydrin, the major cyanogen toxin remaining in poorly processed cassava roots, in HNL overexpressing plants was reduced from days to less than an hour. Thus, allowing for more efficient root cyanogen detoxification. Significantly, the free cyanide generated from the HNL catalyzed hydrolysis of acetone cyanohydrin volatilizes and is not found in cassava foods. In summary, it was possible to elevate the root protein content in a 500 g cassava flour from 11 g in wild-type plants to 33 g in HNL overexpressing plants meeting 48% of the MDR for protein in a single 500 g cassava meal. A cassava meal supplemented with 50 g of soy flour would then meet the MDR for protein in the diet.

2.3 Iron and Zinc Biofortification in Transgenic Cassava

Inorganic micronutrients including iron must be recovered from the soil and stored in readily available forms for the plant. Iron uptake is particularly challenging since ferric iron, the dominant form of oxidized iron in soils, is virtually insoluble in water unlike ferrous iron. To address this challenge, plants use one of two different iron acquisition strategies (Grotz and Guerinot 2006; Takahashi et al. 2001). Dicots (such as cassava) and nongrass monocots utilize a number of processes to enhance the uptake of iron including (1) acidification of the rhizosphere to solubilize ferric iron; (2) reduction of the solubilized ferric iron by a membrane-bound ferric chelate reductase; (3) transport of soluble ferrous iron into the plant root cells by a ferrous iron membrane transporters; and (4) in some plants secretion of flavins to facilitate ferric iron solubilization (Tor-Agbidye et al. 1999; Lanquar et al. 2005; Curie et al. 2009). All four systems are upregulated in roots under conditions of iron deficiency. In contrast, graminaceous plants secrete ferric iron-specific phytosiderophores (PS) derived from methionine and transport the iron-chelation complex into the cell through dedicated transporters (Nozoye et al. 2011). Given that dicot plants transport ferrous iron and that ferrous iron can be toxic through the production of reactive oxygen species mediated by the Fenton reaction, it is critical that ferrous iron be oxidized upon entry into the cell and be stored most frequently as the plastidial iron-storage protein ferritin (4300 Fe atoms/protein with good bioavailability in foods) or to phytic acid (poor bioavailability in foods) (Goto et al. 1999; Coelho et al. 2007; Ravet et al. 2009). Notably, there is virtually no iron stored as phytic acid conjugates in cassava roots.

Three different genetic engineering strategies have been successfully employed to elevate cassava root iron levels. The first approach that demonstrated an increase in

cassava root iron levels was overexpression of the Chlamydomonas iron-specific transporter Fea1 (Narayanan et al. 2011a; Ihemere et al. 2012; Leyva-Guerrero et al. 2012). This unique algal gene is capable of complementing IRT1 iron transporter mutants in Arabidopsis, can transport ferrous iron at high pH (8.5) unlike IRT1, and does not transport toxic heavy metals such as cadmium. Transgenic cassava plants expressing the Fea1 gene under the control of the root specific potato patatin promoter had 3.6-fold higher iron concentrations in roots of greenhouse grown plants. Consistent with the observation that the Fea1 is an iron-specific metal transporter, it was observed that Fea1 transgenic plants had no increase in Zn or Cd content relative to wild-type plants. To determine whether FEA1 expression impacted the expression of other genes involved in regulating iron homeostasis, the expression patterns of multiple genes involved in iron homeostasis in roots, stems, and leaves of wild-type and FEA1 transgenic cassava were assessed. Genes encoding the internal ferric chelate transporter, MeYSL1, and the iron storage proteins, MeFer1, MeFer3, were upregulated in FEA1 transgenic plants relative to wild type, indicating that Fea1 transgenic plants have enhanced capacity for iron mobilization and storage. Consistent with this hypothesis was the observation that leaf MeFer3 expression levels were substantially increased in leaves of Fea1 plants relative to wild-type plants. In contrast, cassava ferric chelate reductase (MeFRO2) expression was downregulated in roots of FEA1 transgenic plants as was observed in Arabidopsis plants that have sufficient iron. These results are consistent with observations in other plants that indicate that whole plant iron homeostasis is transcriptionally regulated and complex. Later field studies comparing the root iron levels of wild-type and Fea1-expressing transgenic plants met with mixed results, however. These results were later attributed to segregation of Fea1 expressing and nonexpressing somaclonal variants of the Fea1 transgenic line.

Narayanan et al. (2015) described a strategy for increasing iron content in transgenic cassava plants by expressing the vacuolar iron transporter Vit1 gene in roots. The AtVIT1 vacuolar iron transporter has been shown to transport Fe into the vacuoles of xylem parenchyma cells in Arabidopsis and participates in regulating whole plant iron homeostasis. Significantly, this transporter is not iron specific and will transport Zn and Cd as well. Root-specific expression of Vit1 resulted in a fourfold increase in root iron levels. However, young transgenic plants expressed chlorosis in emerging leaves. At the molecular level, there was an inverse correlation between Vit1 expression and leaf iron levels, suggesting that expression of Vit1 in roots was disrupting whole plant iron homeostasis. In fact, there was a negative correlation between root and young leaf iron levels, suggesting that iron sequestration in root cell vacuoles disrupted iron transport to leaves. Prussian blue staining for iron indicated high iron concentrations localized in the vascular parenchyma cells in roots in addition to reduced MeFer expression and elevated MeFRO1 expression in leaves indicative of reduced leaf iron content. The reduced iron content in young leaves of Vit1 transgenic plants raises concerns since iron plays a critical role in electron transfer processes in photosynthesis and reductions in leaf iron levels may impair plant growth.

Recently in 2019, an alternative strategy was developed for the simultaneous enhanced uptake of iron and zinc coupled with enhanced ferritin expression in roots to sequester iron. In this study, cassava plants were transformed to express a mutant form of the Arabidopsis IRT1 transporter and the Fer1 gene from Arabidopsis in roots (Narayanan et al. 2019). It was observed that root iron levels were increased 7- to 18-fold relative to wild-type plants to a maximum root iron concentration of 130 μg Fe/g dw. In addition, root Zn levels were elevated by three- to ten-fold to a maximum of 103 μg Zn/g dw. Similar to Vit1 transgenic plants, the iron was sequestered in root vascular parenchyma cells. But in contrast to Vit1 plants, there was no apparent effect on plant growth for plants coexpressing IRT1 and Fer1. In addition, cadmium feeding studies indicated that there was no elevation of cadmium in the transgenic plants relative to controls. Interestingly, iron concentrations in leaves increased nearly fourfold, but leaf zinc concentrations were reduced by as much as twofold. Furthermore, substantial IRT1 and Fer1 gene expression was observed in leaves of transgenic plants, not previously seen with transgenes expressed under the control of the aforementioned gene promoters. One concern remains, however. When transgenic IRT1/Fer1 plants were grown in the field as stake cuttings, there was a statistically significant 30–35% reduction in root yield. Whether this yield loss holds up in clonally (stem) propagated plants from generation to generation will need to be determined by doing repeated multilocational and multigenerational field trials. If the yield loss trait is stable, however, it is unlikely that farmers will adopt an iron and zinc biofortified cassava having a 35% yield loss.

Finally, food processing studies indicated that up to 60% of the elevated root iron and zinc content was retained in the cooked foods made from both transgenic and wild-type plants, indicating that the expression of the transgenes did not impact iron and zinc retention during food processing. These levels of iron and zinc accumulation and retention following food processing suggest that it may be possible to meet 70% of the iron and zinc requirements for children 4–6 years old from a cassava meal. Overall, a biofortification strategy based on using an enhanced common divalent metal transporter coupled with root-specific iron storage protein was shown to effectively address the metal micronutrient nutritional deficiencies of a cassava-based diet.

2.4 Carotenoid Biofortification in Transgenic Cassava and Its Impact on Postharvest Physiological Deterioration

While substantial progress has been achieved in elevating β-carotene levels in cassava cultivars to meet the MDR through breeding efforts, it is arguable that there is still a place for transgenic approaches to more rapidly express high β-carotene traits in cultivars that are farmer preferred and that may take tens of years to breed for elevated β-carotene levels (Ikeogu et al. 2019). Furthermore, there are potential concerns regarding linkage drag between QTLs associated with elevated β-carotene and reduced starch production and plant yields. In 2010, the first transgenic cassava plants were developed with elevated β-carotene levels. The

inspiration for this work was the discovery that there were variant *psy* alleles in natural high β-carotene cultivars of cassava. DNA sequence analysis indicated that there was a R174T mutation in the PSY2-Y-1 high β-carotene allele and an A191D mutation in the PSY2-Y-1 allele. Biochemical studies as well as heterologous gene expression studies in E. coli indicated that the variant PSY2 alleles encoded phytoene synthase enzymes that were catalytically more active than the nonmutant PSY2 alleles present in low β-carotene cassava varieties. Given that phytoene synthase is the rate-limiting enzyme in carotenoid synthesis, it was hypothesized that overexpression of an *E. coli crtB* gene encoding phytoene synthase in all cassava tissues but predominantly in roots using a CP1 promoter would result in elevated root carotenoid levels. In the best-performing CrtB transgenics root, β-carotene levels were increased from 0.41 μg/g dw to 6.67 μg/g dw and total carotenoids increased from 0.65 to 22 μg/g dw. Field trial performance and yield trials were not carried out with these lines, so it is not known whether elevated accumulation of carotenoids impacted biomass yield as observed in cassava varieties bred for enhanced β-carotene levels.

Recently, the previously described strategy to increase carotenoid levels by overexpressing the rate-limiting enzyme phytoene synthase was supplemented by coexpressing in cassava roots the enzyme deoxy-D-xylulose-5-phosphate synthase (DXS), which catalyzes the first-dedicated step in plastidial isoprenoid synthesis, thus potentially addressing any terpenoid substrate limitations for other polyterpenes. Cassava roots harvested from field-grown plants accumulated carotenoids to ≤50 μg/g dw equivalent to a 15- to 20-fold increase in carotenoids relative to roots from wild-type plants. The vast majority of the carotenoids (85–90%) present was all-trans-β-carotene, the most efficient carotenoid for retinol conversion. The accumulation of elevated levels of β-carotene in cassava roots had at least two pleiotropic effects. As previously discussed, there was a 50–60% reduction in dry matter accumulation due to suppression of genes involved in starch synthesis including ADP-glucopyrophosphorylase (AGPase). Similar effects on starch accumulation were observed in potato storage tubers engineered to have elevated levels of β-carotene, indicating perhaps a universal metabolic response to elevated β-carotene levels in unrelated plant species. Significantly, it has been previously demonstrated that overexpression of a modified bacterial AGPase (*glgC* gene) in cassava roots could increase dry matter content by 2.6-fold (Meyer et al. 1998; Ihemere et al. 2006). Given that *E. coli* does not produce β-carotene, it is unlikely that feedback inhibition of the bacterial AGPase by β-carotene will be an issue for transgenic plants coexpressing the *dxs*, *psy,* and *glgC* genes (Gallagher et al. 2003).

In addition, it was observed that roots having elevated β-carotene levels had longer postharvest shelf life than did those from wild-type plants. In the Beyene et al. (2018) study, it was noted that the shelf life of roots with elevated β-carotene levels was greater than 10 days in contrast to wild-type plants that showed symptoms of postharvest physiological deterioration within 5 days and were not marketable by 10 days after harvest. Cassava root PPD has previously been shown to be initiated by cyanide poisoning of mitochondrial electron transport, leading to the production of reactive oxygen species (ROS) and the induction of programmed cell death

pathways (Buschmann et al. 2000; Fath et al. 2002; Huang et al. 2001; Fernando Cortés et al. 2002; McDonald et al. 2002; Robson and Vanlerberghe 2002; Isamah et al. 2003; Reilly et al. 2007; Liu et al. 2017; Qin et al. 2017). Plant mitochondria, however, also have a cyanide-insensitive alternative oxidase that is activated under stress reducing the generation of ROS but also reducing the efficiency of ATP synthesis (Gonzalez-Meler et al. 1999; Albury et al. 2002; Moore et al. 2002). Transgenic cassava roots expressing a plant mitochondrial and cyanide-insensitive alternative oxidase (AOX) were shown not to produce elevated levels of ROS and had root shelf lives that extended 3 weeks beyond harvest date (Zidenga et al. 2012). Given the central role of ROS in the PPD process, it is not surprising that elevated β-carotene levels would reduce PPD and extend cassava root shelf life (Sanchez et al. 2006). β-Carotene is a well-known physical and chemical quencher of ROS (Martin et al. 1999; Ohmiya et al. 2006). Not unexpectedly, however, two of three AOX transgenic lines tested had substantial reductions in root yield when grown in the field. Reductions in root yield were not observed, however, in seven independent AOX lines when grown under greenhouse conditions for 4 months. In fact, the root yields of the seven independent AOX transgenic plants were nearly double that of wild-type plants. This reduction in root yields in older field grown plants may reflect the reduction in ATP generating capacity in engineered mitochondria expressing AOX relative to mitochondria utilizing full chain electron transport and proton coupled ATP synthesis (Moller 2001; Millenaar and Lambers 2003). These results suggest that ATP demands may change with root age and growth conditions impacting growth. This is not surprising given the central role of ADP-glucose in starch synthesis.

The potential for β-carotene degradation by ROS generated during PPD has yet to be assessed. It has been demonstrated, however, that as little as 24 h exposure to sunlight can reduce β-carotene levels by 75–90% in cassava chips. Thus, any attempt to enhance cassava β-carotene levels must be coupled with the appropriate food processing technology to avoid substantial β-carotene losses (Eyinla et al. 2018). Finally, can elevated β-carotene levels in cassava foods be efficiently assimilated (De Moura et al. 2015; Talsma et al. 2013, 2016)? In studies using transgenic plants having greater than a tenfold increase in β-carotene levels, it was demonstrated that for most food processing technologies except gari production that at least a 70% of the β-carotene present in the unprocessed root was retained in the food product. Notably, gari production includes a roasting step at 19 °C. Most importantly, β-carotene concentrations in human Caco-2 cells fed *in vitro* digested and micellarized cassava foods were 57–73, 42–83, and 49–83 times greater after exposure to *in vitro* digested boiled cassava, fufu, and gari, respectively, prepared from the transgenic cassava roots having elevated β-carotene levels compared to identically processed wild-type roots (Failla et al. 2012). These results again demonstrate that food preparation procedures that avoid exposure to sunlight and heat are critical for retention of bioavailable β-carotene and that elevated levels of β-carotene are bioavailable.

3 Summary

Substantial progress has been achieved in reaching the original programmatic goals of the HarvestPlus and BioCassava Plus programs of providing sufficient protein and micronutrients in traditionally bred and engineered cassava varieties to meet the needs of Sub-Saharan Africans who depend on cassava foods (Andersson et al. 2017). Challenges for cassava biofortification remain, however (Mutuku et al. 2020). Engineering or breeding elevated micronutrient levels into cassava roots have been shown to result in reductions in starch and dry matter yields in the field. In some cases, the molecular basis for these reductions in yield is beginning to be resolved and points toward mitigation strategies including elevating the root-specific activity of AGPase activity to increase starch accumulation. This strategy may be most effective when using recombinant bacterial enzymes (*glgC*) that have been engineered to limit allosteric feedback inhibition. Substantive progress on cassava biofortification using breeding approaches, however, has been limited to only increases in β-carotene levels. It is not clear yet if there is sufficient genetic variation in cassava to breed for substantially enhanced iron, zinc, or protein content.

The ability to genetically engineer this clonally propagated plant offers significant advantages for accelerating the generation of biofortified cassava, particularly biofortified user-preferred varieties. The time to go from the lab to field trials for a genetically engineered farmer-preferred cassava variety is 2 years in contrast to 6–8 years for a new fixed-trait variety developed through accelerated breeding programs. Regardless, there is a critical need for both technology platforms to realize the full potential of biofortified cassava and to learn from each approach how to more intelligently and rapidly develop new products with stacked and complex traits in farmer-preferred varieties. The challenge now is to move progress forward. The unfortunate realization is that progress toward a complete biofortified cassava having adequate protein iron, zinc, and β-carotene has slowed in the last 10 years. This challenge is clearly the result of less research and development funding in this sector and the failure to establish well-funded centers of excellence in cassava metabolic phenotyping and engineering. Given the fact that undernourishment and malnutrition have been increasing in SSA since 2041 and are likely to increase further by 2030, it is imperative that the global food security community address the need for more nutritious and higher-yielding cassava cultivars.

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Transgenics for Biofortification with Special Reference to Rice

17

Shuvobrata Majumder, Karabi Datta, and Swapan Kumar Datta

Abstract

Biofortification is the process of making nutritionally enhanced varieties of food crops through different approaches including agronomic and innovative breeding. It is an easy, affordable, and effective approach to combat micronutrient-related malnutrition or hidden hunger. Vitamin A deficiency (VAD), iron deficiency anaemia (IDA), and zinc deficiency are forms of hidden hunger and cause serious health problems in two billion people worldwide. Staple cereals are the most accessible and contributed most in daily share of energy intake for the population. Rice, wheat, and maize are the top highest producing cereals. Making available with biofortified crops will serve most of the people to stay healthy and overcome the hidden hunger. Successful attempts have been made to develop cereals biofortified with provitamin A, iron, and zinc. In this chapter, we have highlighted some of these biofortification strategies with special focus on rice, the most consumed staple crop in the world.

Keywords

Biofortification · Hidden hunger · Provitamin A biofortification · Iron biofortification · Zinc biofortification · Transgenic crops

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1 Introduction

1.1 Hidden Hunger and Nutritional Security

Food and nutritional security is now a global priority to feed the continuously growing world population. Currently, there are 7.79 billion people in the world and two billion of them suffer from nutritional insecurity in the form of micronutrient deficiency malnutrition or hidden hunger (https://population.un.org/wpp/Download/Standard/Population, accessed 24 August 2020). Nutrient deficiency has affected one out of three people, mostly from Africa, South Asia, and Latin America (FAO 2015). Hidden hunger slowly weakens the immune system, stunts human growth (physical and mental), and even causes death. Everyday, more than 24,000 people die from 'hidden hunger' and malnutrition worldwide (Fiaz et al. 2019). Vitamin A deficiency (VAD), iron deficiency anaemia (IDA), and zinc deficiency are ubiquitous and cause serious health problems worldwide (Fig. 17.1). Preschool children and pregnant women suffer most from hidden hunger.

Vitamin A deficiency (VAD) is a leading cause of blindness in children which if not treated can cause permanent blindness with other serious illnesses. VAD is also responsible for increased rates of childhood and maternal mortality. VAD prevalence rate in children under the age of 5 was reported to be the highest (60–70%) in South Asia and sub-Saharan Africa, where an 85% prevalence rate in Kenya made it the highest in the world (WHO 2009) (Fig. 17.1a). The prevalence rate of VAD in pregnant women is 15–20% in South Asia and sub-Saharan Africa and 20–25% in Central/East Asia, North Africa, and the Middle East, along with higher rates across Asia and Africa (WHO 2009) (Fig. 17.1b).

The World Health Organization (WHO) estimated that anaemia affects around 800 million children and women and causes 20% of the maternal deaths (WHO 2015). People from Southeast Asia, Eastern Mediterranean, and African Regions had the lowest mean blood haemoglobin concentrations in the world population. WHO's Nutrition Landscape Information System (NLiS) data on Asian countries showed that Pakistani children were the most anaemic (61%) in 2011, and India had 51.5% anaemic pregnant women in 2016. Developing countries suffer more from the IDA than the higher-income regions like North America, Europe, and some part of Central Asia (Fig. 17.1c).

Zinc is essential for human health, and its deficiency causes hidden hunger. It is involved in homeostasis, immune responses, oxidative stress, apoptosis, and aging (Chasapis et al. 2012). It serves as a catalytic, structural, and regulatory ion. Zinc deficiency in humans affects the immune system, skeletal system, central nervous system, gastrointestinal, and reproductive system (Jurowski et al. 2014). Children and pregnant women are most vulnerable to zinc deficiency. The prevalence of zinc deficiency varies between 15 and 50% across sub-Saharan Africa and South Asia, and about 17% of the world's population is at risk of inadequate zinc intake (Fig. 17.1d) (Wessells and Brown 2012).

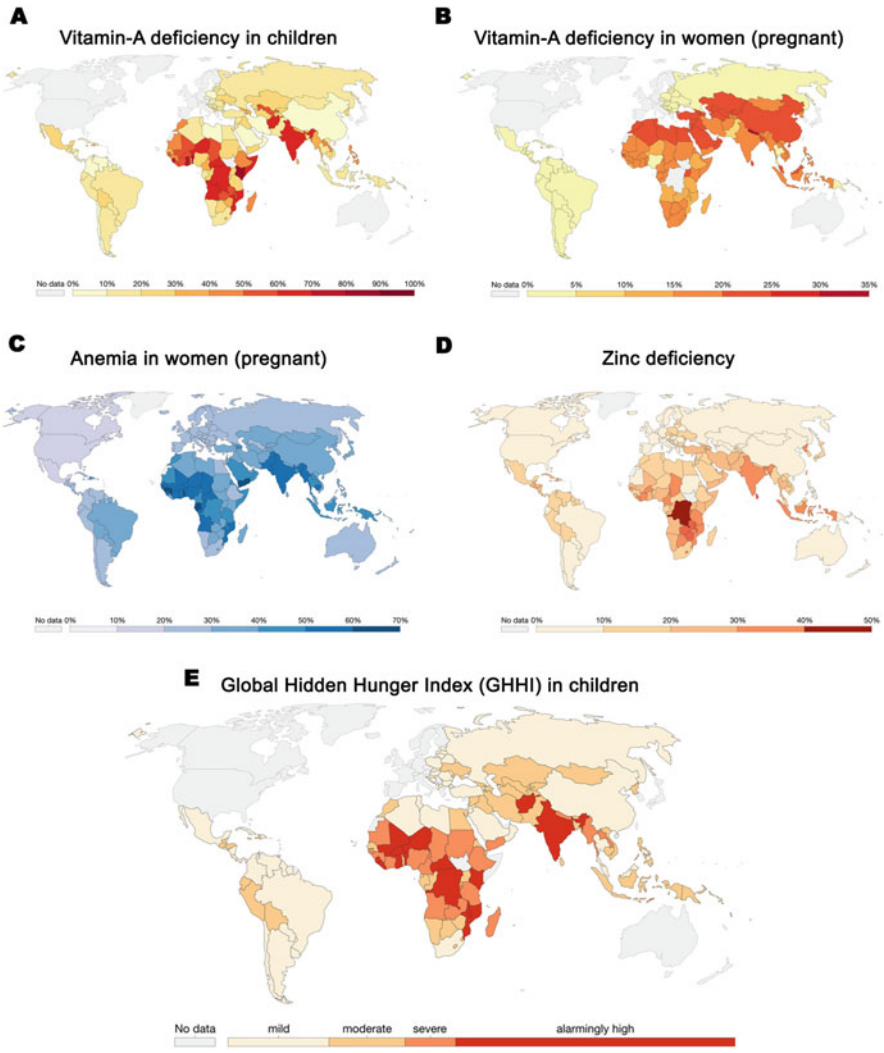

Fig. 17.1 Hidden hunger worldwide. (**a**) Prevalence of vitamin A deficiency (VAD) in children. (**b**) Prevalence of VAD in pregnant women. (**c**) Prevalence of iron deficiency anaemia (IDA) in pregnant women. (**d**) Global prevalence of zinc deficiency. (**e**) Global Hidden Hunger Index (GHHI) in preschool children. (Adapted from online resources https://ourworldindata.org/micronutrient-deficiency, accessed on August 5, 2020)

1.2 Hidden Hunger Index and Staple Crops

Measuring hidden hunger from a single parameter would be inadequate because multiple micronutrient deficiencies combindly could affect health conditions. One widely accepted way to measure the severity of hidden hunger is the Global Hidden

Hunger Index (GHHI). This was based on the nutritional indicators in preschool children in 149 countries. The GHHI calculations are based on national prevalence data on stunting, VAD, and vitamin A levels (Muthayya et al. 2013). The GHHI is 'alarmingly high' in sub-Saharan Africa and South Asia; 'mild' in Central Europe and East Asia, North Africa, and Latin America; and 'moderate-to-severe' in the rest of the world (Fig. 17.1e).

A large population of the world is suffering from hidden hunger. Their food is not enough to supply an adequate amount of essential micronutrients. Essential micronutrients can be received by three processes—supplementation, food fortification, and biofortification (Miller and Welch 2013). Supplementation is the process of delivery of essential micronutrients in different forms like pills, powder, and liquid. Food fortification deals with food processing, by which a small amount of micronutrients is mixed with commonly consumed foods like cereals and pulses. Biofortification is the process of increasing the micronutrients in food crops through agronomic and breeding approaches (Hannah 2017).

Biofortification of staple crops could be promising in curbing hidden hunger since staple crops (including roots and tubers) contribute to a major share of energy intake (daily calories). It has been observed that Asians and Africans get 60–80% of their daily energy requirement from staple crops (Fig. 17.2a). Cereals are the majorly produced staple crops followed by coarse grains (staple crops except rice and wheat), olicrops, pulses, roots and tubers, vegetables, and fruits (Fig. 17.2b). Rice (*Oryza sativa*), wheat (*Triticum aestivum*), and maize (*Zea mays*) are the most popular sources of daily energy intake (Fig. 17.2c). These three cereals have been produced all over the world (Fig. 17.2d). It is understandable that biofortification of these three cereals holds the possibility to deliver the micronutrients to a larger population.

Biofortification through plant breeding can be of two types—conventional breeding and molecular breeding. In this chapter, molecular breeding approaches have been discussed that are used to introduce nutritionally important, beneficial genes into crops, overcoming species barriers via the transgenic approach. We have extensively focused on rice which contributes to the majority (up to 70%) of daily calories from staple food for more than half of the world's population (Mishra et al. 2018).

2 Rice Biofortification

2.1 Provitamin A Biofortification in Rice (Golden Rice)

Provitamin A fortified rice is popularly known as golden rice. The golden colour of the grain is due to the seed-specific expression of β-carotene. Golden rice development was a successful example of metabolic engineering where genes from different sources (plants and bacteria) have been successfully introduced into rice (Fig. 17.3a). The phytoene synthase (*PSY*) gene from daffodil (*Narcissus pseudonarcissus*) and the phytoene desaturase (*CRTI*) gene from bacteria *E. uredovora* were introduced in japonica rice variety Taipei-309 to develop the carotenoid-rich rice (Burkhardt et al.

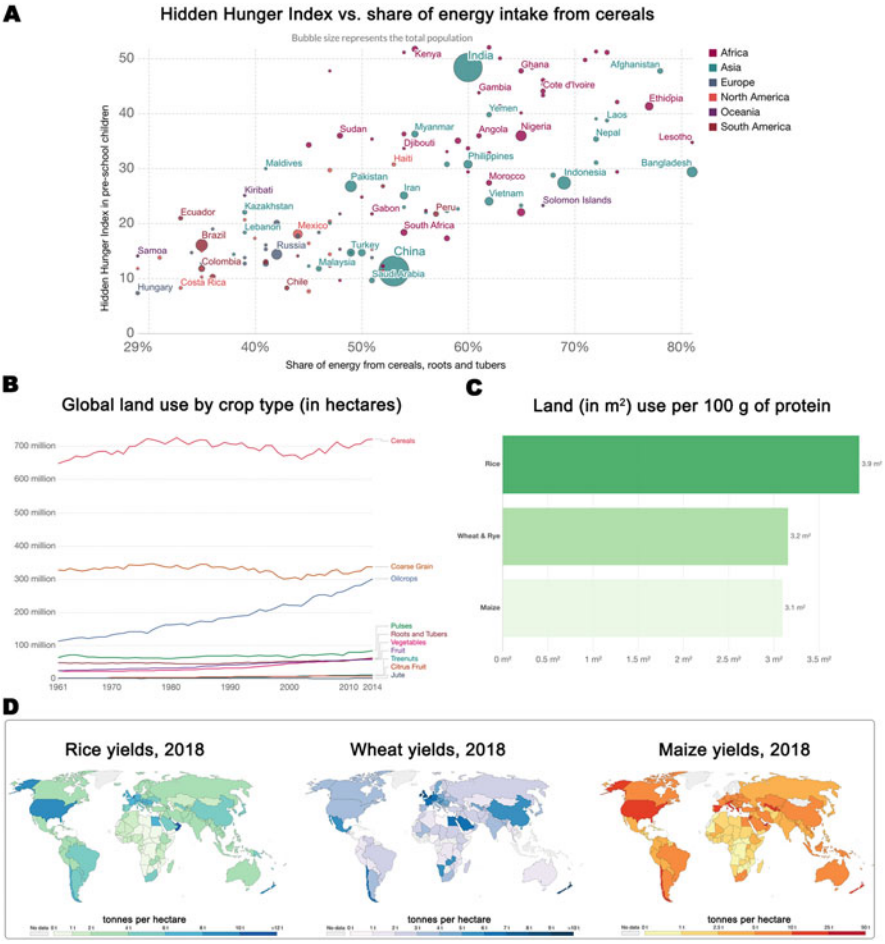

Fig. 17.2 Global statistics. (**a**) Hidden hunger index in preschool children in relation with energy intake from cereals including roots and tubers and country-wise population. (**b**) Global agricultural land used by major crops. (**c**) Land used per 100 g protein production. (**d**) Global yields of rice, wheat, and maize according to 2018 data. (Adapted from online resources https://ourworldindata.org/micronutrient-deficiency, accessed on August 5, 2020)

1997). These genes were expressed under the glutelin promoter to ensure that the carotenoids are produced and retained in the endosperm even after the grain milling processes (Ye et al. 2000). Golden rice (Taipei-309) produced 1.6 μg/g total carotenoids. These genes were also introduced in indica rice varieties which then produced 2.32 μg/g β-carotene (provitamin A) in polished IR64 rice and 3.92 μg/g in BR29 golden rice (Fig. 17.3b) (Datta et al. 2006). The search for candidate genes for more carotenoids production in rice grains continues, and many successes have been reported (Table 17.1). Golden rice-2 was developed using maize *ZmPSY* and *E. uredovora CRTI* genes which produced 37 μg/g of total carotenoids in its grains

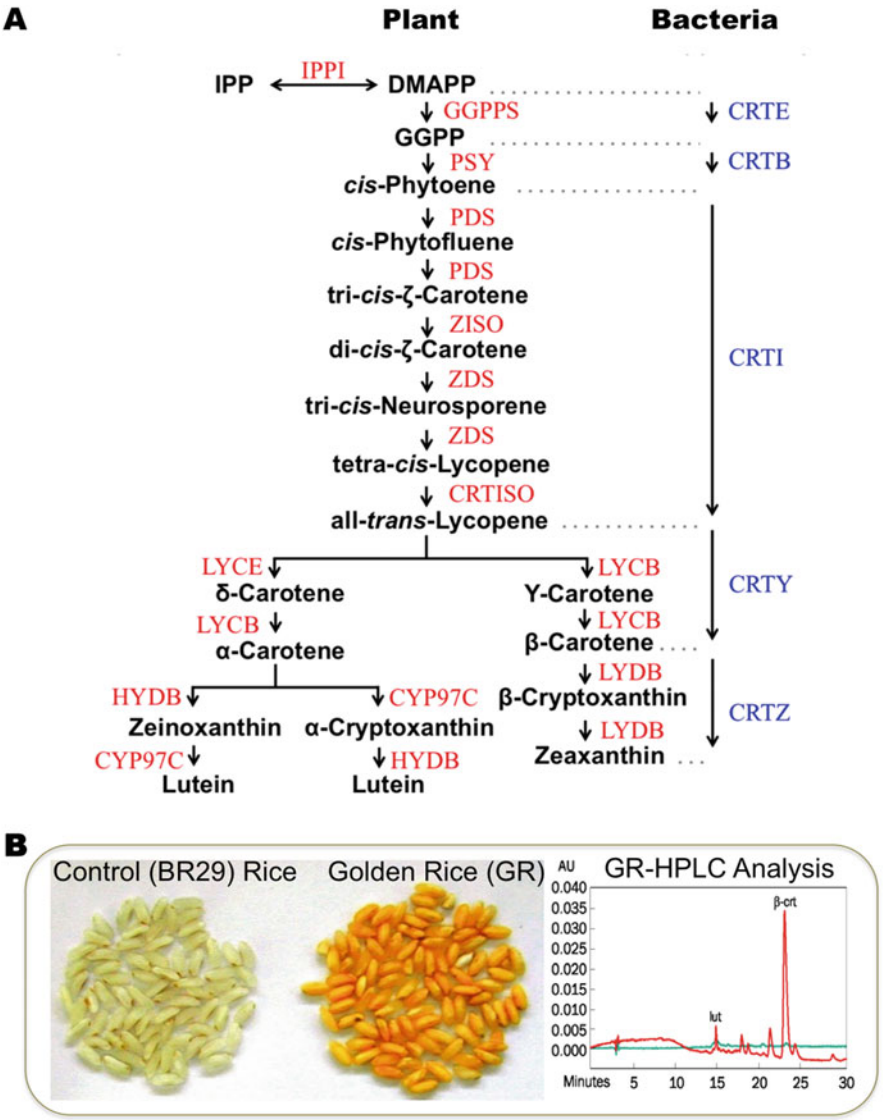

Fig. 17.3 Indica golden rice (GR) development. (**a**) Carotenoid biosynthesis pathway in plants with equivalent steps in bacteria. Enzymes involved in plants are in red colour and in bacteria are marked in blue colour. *IPPI* isopentenyl diphosphate isomerase; *DMAPP* dimethylallyl diphosphate; *IPP* isopentenyl diphosphate; *GGPP* geranylgeranyl diphosphate; *GGPPS* GGPP synthase; *PSY* phytoene synthase; *PDS* phytoene desaturase; *ZISO* ζ-carotene isomerase; *ZDS* ζ-carotene desaturase; *CRTISO* carotenoid isomerase; *LYCB* lycopene β-cyclase; *LYCE* lycopene ε-cyclase; *CYP97C* carotene ε-ring hydroxylase; *HYDB:* β-carotene hydroxylase; *CRTE*: bacterial GGPP synthase; *CRTB*: bacterial phytoene synthase; *CRTI* bacterial phytoene desaturase/isomerase; *CRTY* bacterial lycopene cyclase; *CRTZ* bacterial β-carotene hydroxylase (Ghosh et al. 2019), (**b**) golden indica BR29 (right) and its control rice (left). The transgenic golden rice line shows expression of β-carotene in rice seeds; measured by HPLC analysis. The HPLC curves in red

(Paine et al. 2005). A combination of *ZmPSY1*, *PaCRT1* (bacterial), *AtDXS* (gene for continuous supply of metabolic precursors), and *AtOR* (gene for formation of a metabolic sink) was used to develop golden rice which was able to produce up to 31.78 μg/g total carotenoids in rice grain (Bai et al. 2016). During the development of golden rice, marker genes were used as routine experimental processes, but marker-free crops would be a more desirable option (Majumder et al. 2019). As demonstrated by Parkhi et al. (2005) and Baisakh et al. (2006), marker genes can be successfully removed from GR, thereby making it 'marker free'.

Golden rice development through introgress lines by breeding (Baisakh et al. 2006) and development through dihaploid (DH) homozygosity was reported (Datta et al. 2014). With the help of DH homozygosity approaches, isogenic GR lines can be developed rapidly through anther or pollen culture from existing golden rice lines. The amount of carotenoids in golden rice grains degrades upon storage. In the seed, molecular oxygen is inserted into polyunsaturated fatty acids with the help of lipoxygenase (LOX) enzyme which oxidizes carotenoids and causes its deterioration (Carrera et al. 2007). Fourteen types of *LOX* genes have been identified in the rice genome; among them, *r9-LOX1* causes carotenoid deterioration (Gayen et al. 2015). The RNAi-mediated downregulation of *r9-LOX1* gene ameliorated carotenoid deterioration in golden rice (Gayen et al. 2015). This silencing strategy could be effective for improving seed quality and long storage of golden rice.

2.2 High Iron Rice

Molecular breeding for high iron rice development involves many strategies that have been taken under iron biofortification research projects. Here a few popular strategies for rice iron biofortification have been highlighted.

2.2.1 Expression of the *Ferritin* Gene

Ferritin is a large multi-subunit protein with ferroxidase activity and the capability of storing up to 4500 iron atoms in a complex form (Andrews et al. 1992). This complex is not toxic, and the human intestine can absorb iron from this iron complex. Soybean *ferritin* genes—*SoyferH1* and *SoyferH2*—are well studied and are popularly used for iron biofortified rice development (Kok et al. 2018). To fight IDA, many iron biofortified rice varieties have been developed using the soybean *ferritin* genes, expressed through seed-specific promoters like globulin and glutelin, and gave results of 3.7-fold iron increase in rice grain (Goto et al. 1999; Lucca et al. 2002; Vasconcelos et al. 2003; Qu et al. 2005; Khalekuzzaman et al. 2006; Paul et al. 2012; Oliva et al. 2014). Such high iron rice varieties have been part of many introgressed breeding projects for iron biofortification in rice in local cultivars.

Fig. 17.3 (continued) colour for polished seeds sample and in blue colour for unpolished golden rice seeds. (Source: Datta et al. 2007)

Table 17.1 Selected reports on golden rice development (Modified from Datta and Datta 2020)

No.	Genes involved	Rice variety	Result	References
1.	Daffodil (*Narcissus pseudonarcissus*) phytoene synthase, *psy* gene	japonica Taipei 309 variety	Formation of carotenoid-specific intermediate phytoene	Burkhardt et al. (1997)
2.	Phytoene synthase (*psy*) and *β*-cyclase (*lcy*) from *Narcissus pseudonarcissus* and bacterial (*E. uredovora*) phytoene desaturase (*CRTI*)	Japonica rice cultivar TP 309	Total carotenoids—1.6 μg/g	Ye et al. (2000)
3.	Phytoene synthase (*psy*) from daffodil, with seed-specific glutelin promoter (Gt-1), bacterial phytoene desaturase (*crtI*) fused to the transit peptide sequence of the pea-Rubisco small subunit were driven by the constitutive promoter CaMV35S and lycopene β-cyclase (*lcy*)	IR 64, BR 29 Nang Hong Cho Dao and Mot Bui	Total carotenoids—1.05 μg/g in T_1 seeds in Nang Hong Cho Dao	Datta et al. (2003)
4.	Phytoene synthase (*psy*) from *Narcissus pseudonarcissus* and bacterial phytoene desaturase (*crt*I)	Japonica rice cultivar (Taipei 309) and Indica rice cultivars (IR64 and MTL 250)	Total carotenoids in T_2 grains of Taipei 309 and IR64 are 1.2 μg/g and 0.8 μg/g	Hoa et al. (2003)
5.	Maize phytoene synthase (*Zmpsy*) and bacterial (*E. uredovora*) phytoene desaturase (*crt*I) gene	Asanohikari rice cultivar	Total carotenoids—37 μg/g	Paine et al. (2005)
6.	Introgression line with phytoene synthase (*psy*) and phytoene desaturase (*crt*I) and lycopene β-cyclase (*lcy*)	Indica rice cultivar IR64	Total carotenoids—1.06 μg/g	Baisakh et al. (2006)
7.	Phytoene synthase (*psy*) and phytoene desaturase (*crt*I)	Indica rice cultivars (IR 64 and BR 29)	Total carotenoids in BR 29 and IR 64 are 9.34 μg/g and 2.32 μg/g	Datta et al. (2006)
8.	Phytoene synthase (*psy*) and phytoene desaturase (*crt*I)	Indica rice cultivar	Total carotenoids—6.77 μg/g	Rai et al. (2007)
9.	Another culture-derived line with phytoene synthase (*psy*) *Narcissus pseudonarcissus* and	Indica rice cultivar BR 29	Total carotenoids—3.188 μg/g	Datta et al. (2014)

(continued)

Table 17.1 (continued)

No.	Genes involved	Rice variety	Result	References
	phytoene desaturase (*crt*I) *E. uredovora*			
10.	(a) Co-expressed *AtDXS*, *ZmPSY*1, and *PaCRT*I (b) Co-expressed *AtOR*, *ZmPSY*1, and *PaCRT*I	Wild-type rice (*Oryza sativa* L. cv. EYI105)	(a) Total carotenoids—31.78 μg/g (b) Total carotenoids—25.83 μg/g	Bai et al. (2016)

The *ferritin* genes from IR68144 high iron rice (Vasconcelos et al. 2003) have been introgressed into Swarna, a popular indica cultivar which produces 2.54-fold more iron in milled rice grain as compared to control Swarna (Paul et al. 2014).

2.2.2 Chelation-Based Strategy

Roots of rice and other graminaceous staple crops are secret soluble phytosiderophores (PS) like mugeniec acid (MA) and avenic acid. PS are small organic compounds secreted from the plant roots under low iron concentration. They have a high-affinity chelating property for iron or zinc (Romheld and Marschner 1990; Marschner and Romheld 1994). Increasing the expression of PS could increase the iron uptake in plants, and this strategy has been applied for high iron rice development. In rice, nicotianamine synthase (NAS) and nicotinamide transferase are the two main enzymes involved in the release of PS (Huguchi et al. 1999; Nozoye et al. 2011). Overexpression of the rice NAS-producing genes (*OsNAS1*, *OsNAS2*, and *OsNAS3*) was implemented to develop high iron rice (Lee et al. 2009, 2012; Johnson et al. 2011). Barley *NAS* gene (*HvNAS1*) was expressed for high iron rice development (Masuda et al. 2009). Barley's *IDS2* and *IDS3* genes produce special types of MA which have better stability with Fe^{3+} than the rice, in slightly acidic soil (Von-Wiren et al. 2000). Expression of the IDS3 gene in rice increases iron concentration in polished rice grains (Suzuki et al. 2008; Masuda et al. 2008). This chelation-based strategy doubled the iron concentration in polished biofortified rice.

2.2.3 Iron Influx in Rice Grains

Metal transporters play a vital role in iron influx in rice grains. A few such iron transporter genes have been expressed in rice for iron biofortification. The yellow stripe-like (*YSL*) genes play an important role as iron transporters in the rice endosperm (Ishimaru et al. 2010; Koike et al. 2004). Overexpression of the *OsYSL2* gene increased fourfold iron concentration in polished grain (Ishimaru et al. 2010).

Another approach is retention of iron in the seeds by limiting iron distribution in other plant parts. One such example is blocking (knockout) of the rice vacuolar iron transporter (*VIT*) genes. Another example is the knockout of the *OsVIT1* and *OsVIT2*

genes resulting in 1.4-fold increased iron accumulation in rice grain (Zhang et al. 2012).

2.2.4 Silencing of Phytic Acid in Rice Seed

In cereals, phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate or IP6 or $InsP_6$) accumulates (up to 85%) in the aleurone layer of grains (Raboy 1997). It is accumulated as a salt mixture called phytate. Phytate is a potent chelator of divalent ions like iron (Fe^{2+}), zinc (Zn^{2+}), magnesium (Mg^{2+}), and calcium (Ca^{2+}) and reduces the bioavailability of these ions. Phytic acid or phytate can be reduced via RNAi-mediated gene silencing of the phytic acid pathway genes (Fig. 17.4). The first enzyme of rice phytic acid biosynthesis pathway is myo-inositol-3-phosphate synthase (MIPS) (Suzuki et al. 2007). MIPS was targeted to lower down phytate in rice grains. This enzyme was silenced in rice by using the constitutive promoter CaMV35S (Feng and Yoshida 2004) and seed-specific promoters—glutelin B-1 (Kuwano et al. 2006, 2009) and oleosin 18 (Ali et al. 2013a). Genetic-modified (GM) rice showed lower myo-inositol level resulting in low phytic acid

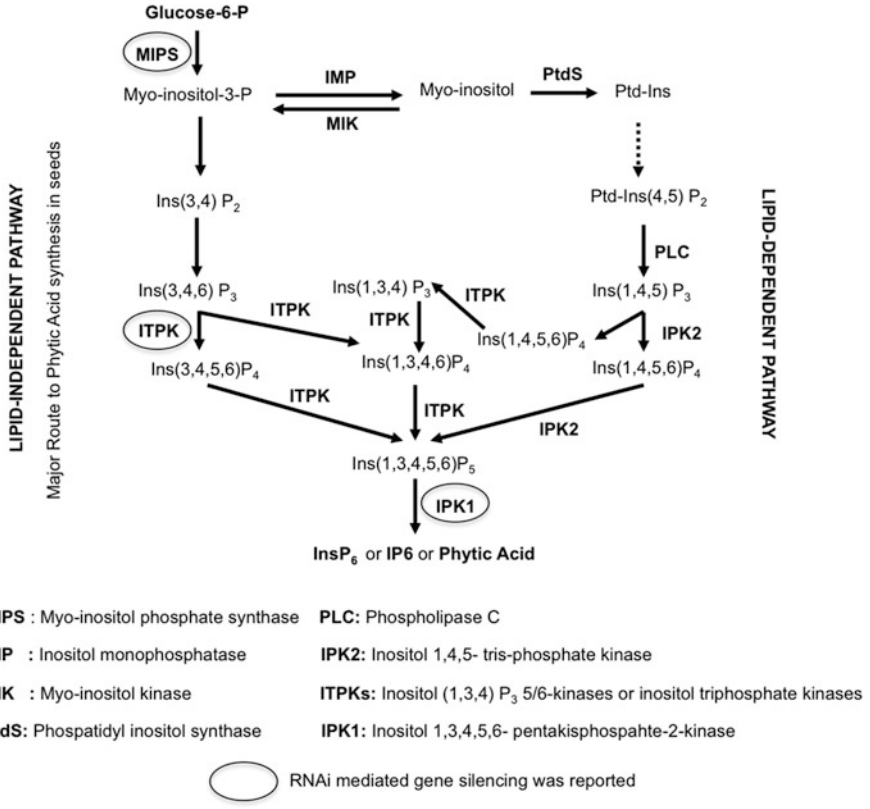

Fig. 17.4 Rice phytic acid metabolism pathway (source: Majumder et al. 2019)

concentration in grains. Two other enzymes—inositol triphosphate kinases (ITPK) and IPK1 of the phytic acid pathway − have been silenced. The homolog of *ITPK* gene (*OsITP/6K-1*) has been silenced in indica rice cultivar Khitish and found with 1.3-fold higher iron accumulation with higher zinc (1.6-fold) and inorganic phosphate (3.2-fold) in rice gains (Karmakar et al. 2020). The final step enzyme, IPK1, has been silenced in Pusa Sugandhi II (PSII) using oleosin 18 promoter which leads to 1.8-fold more iron accumulation in rice grains (Ali et al. 2013b). In these RNAi-based silencing of ITPK and IPK1 enzymes, no alteration in plant growth, development, and seed germination have been observed (Karmakar et al. 2020; Ali et al. 2013b).

2.2.5 Release of Iron from Phytic Acid

Release of chelated minerals (i.e. Fe^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+}), including phosphate, from phytic acid or phytate is a vital part of the biofortification strategy to improve bioavailability of ions. Humans and other monogastric animals possess negligible or no phytase activity in the digestive tract; as a result, they can degrade only about 10% of the ingested phytate (Colombo et al. 2020). Endosperm-specific expression of phytase catalyses the hydrolysis of phytic acid or phytate, thereby improving mineral bioavailability (Welch 2002). Expressing the fungal (*Aspergillus fumigatus*) phytase gene (*Afphytase*) in rice was found to be promising (Wirth et al. 2009; Boonyaves et al. 2016, 2017). Bacterial (*E. coli*) phytases gene *appA* was tested in the Khitish indica rice cultivar and found that it increased twofold iron, threefold zinc, and fourfold inorganic phosphorus (Pi) level in grains (Bhattacharya et al. 2019).

2.2.6 Metallothionein (MT) to Improve Iron Bioavailability

GM Taipei-309 rice was developed by introducing a cysteine-rich MT gene with *Afphytase* gene (Lucca et al. 2001). Each plant source MT molecules contains 12–74 cysteines which facilitate iron (non-haem) absorption (Taylor et al. 1986; Hsieh et al. 1995). Endosperm-specific expression of MT gene in combination with *Afphytase* gene results in 130-fold phytase increase and complete degradation of phytic acid. This approach can increase iron bioavailability in biofortified rice.

2.2.7 Multi-gene Approach for Iron Biofortification in Rice

Different combinations of genes have been tested for iron biofortified GM rice development. GM Taipei-309 rice was developed by introducing *Pvferritin*, *AtNAS1*, and *Afphytase* genes which gave 6.3-fold increased iron accumulation in polished grain (Wirth et al. 2009). Another GM Taipei-309 rice was reported to harbour the *AtIRT1*, *Pvferritin*, *AtNAS1*, and *Afphytase* genes and produce 4.3-fold more iron in polished grain (Boonyaves et al. 2016). Tsukino Hikari rice was developed through the introduction of *SoyferH2*, *HvNAS1*, and *OsYSL2* genes and was able to produce sixfold increased iron accumulation in brown rice (Masuda et al. 2012). Iron biofortified IR-64 were developed by expressing *SoyferH1* and *OsNAS2* gene combinations which produced sixfold higher iron in polished seeds (Trijatmiko et al. 2016). GM Paw San Yin iron biofortified rice was produced by introducing the

SoyferH2, *HvNAS1*, and *OsYSL2* gene combination which produced 3.4-fold higher iron in polished seeds (Aung et al. 2013). GM Nipponbare had the *AtIRT1*, *Pvferritin*, and *AtNAS1* gene combination and produced 4.7-fold higher iron in polished seeds (Boonyaves et al. 2017). GM Tsukino Hikari biofortified rice was developed using the *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *SoyferH2* gene combination and found to give fourfold higher iron accumulation in polished grain (Masuda et al. 2013). In the future, a new combination of genes could facilitate better iron accumulation and superior iron biofortified rice varieties.

2.3 High Zinc Rice

In plants, zinc uptake and homeostasis are similar to that of iron; therefore, zinc biofortification strategies closely resemble iron biofortification strategies. In rice, many ZIP family transporter proteins facilitate both iron and zinc uptake and homeostasis. Overexpression of such ZIP family *OsIRT* and *MxIRT* genes increased iron and zinc concentrations in rice (Lee and An 2009; Tan et al. 2015). Arabidopsis *IRT1* and *NAS1* genes with *Pvferritin* expression in rice increased zinc accumulation in seeds with 4.7-fold increased iron (Boonyaves et al. 2017). Similar result was observed when *Afphytase* was incorporated with the three genes *AtIRT1*, *AtNAS1*, and *Pvferritin* in rice (Boonyaves et al. 2016). Overexpression of the *OsNAS* gene can substantially increase iron and zinc accumulation in rice grains (Johnson et al. 2011; Lee et al. 2012). Expression of the *MxIRT1* gene in transgenic rice exhibited a threefold increase in iron and zinc accumulation in seeds (Tan et al. 2015).

Some strategies for iron biofortification also increased zinc accumulation in rice grains. Expression of the *ferritin* gene increased zinc accumulation along with iron in rice seeds. Expression of the *Osfer2* gene in PSII rice cultivar increased 1.37-fold zinc concentration with 2.09-fold iron (Paul et al. 2012). Silencing of phytic acid metabolic pathway genes increases zinc accumulation in rice grains. RNAi-mediated silencing of the *MIPS* (Ali et al. 2013a), *IPK1* (Ali et al. 2013b), and *ITPK* genes (Karmakar et al. 2020) of the phytic acid metabolic pathway increased zinc concentration in rice grains. This could be due to the natural synergistic effect present in plants for iron and zinc.

2.4 High Oleic Rice

Rice bran oil (RBO) is consumed in many Asian countries and other parts of the world as a 'healthy cooking oil' as it contains tocopherols, tocotrienols, phytosterol, and γ-oryzanol (Sohail et al. 2017). RBO is mainly composed of 13–22% palmitic acid (16:0, saturated), 37–52% oleic acid (18:1, monounsaturated), and 27–40% linoleic acid (18:2, polyunsaturated) (Taira et al. 1988). Among these fatty acids, oleic acid (18:1) has better oxidative stability and helps prevent hypertension (high blood pressure), coronary heart disease (heart attack), cerebrovascular disease

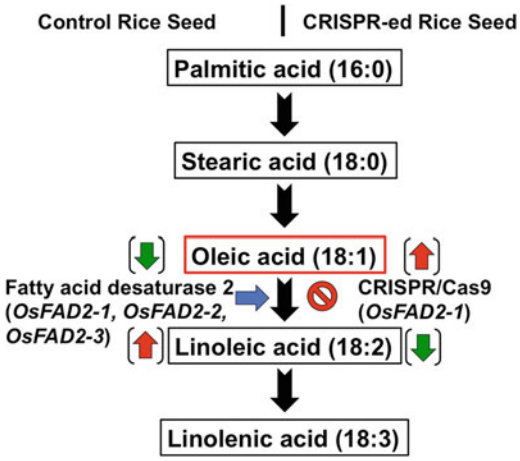

Fig. 17.5 The CRISPR/Cas9 genome editing approach to produce high oleic/low linoleic rice seeds

(stroke), peripheral vascular disease, and other cardiovascular diseases (Lopez-Huertas 2010).

In the rice seed, oleic acid is converted to linoleic acid by the fatty acid desaturase 2 (FAD2) enzyme (Okuley et al. 1994) (Fig. 17.5). Four FAD2 genes were identified in the rice genome, designated as *OsFAD2-1*, *OsFAD2-2*, *OsFAD2-3*, and *OsFAD2-4* (Zaplin et al. 2013). The *OsFAD2-4* is considered as nonfunctional; the *OsFAD2-1* gene is highly expressed in rice seeds; and the *OsFAD2-2* and *OsFAD2-3* are expressed specifically in rice roots (Abe et al. 2018). RNAi-mediated silencing of the *OsFAD2-1* gene expression increased oleic acid concentration in rice seeds (Tiwari et al. 2016; Zaplin et al. 2013). The new genome editing tools—clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas9) (CRISPR/Cas9)—have been applied to knock out *OsFAD2-1* gene from the Nipponbare rice genome to increase oleic acid content in rice seeds (Abe et al. 2018). In the CRISPR-ed rice, the oleic acid content increased twice that of wild type, and linoleic acid decreased to undetectable levels in brown rice seeds (Fig. 17.5). This CRISPR/Cas9 genome editing approach to produce high oleic/low linoleic rice seeds could contribute immensely towards the commercial production of improved RBO.

3 Wheat Biofortification

Wheat is the second highest produced cereal crop (Fig. 17.2c). Successful molecular breeding approaches for wheat biofortification are limited compared to rice. Provitamin A (carotenoids) have been expressed in wheat which closely resembles golden rice biofortification. The maize *psy1* was expressed with a bacterial (*E. uredovora*) *crtI* gene in an elite wheat variety EM12 to produce carotenoids (Cong et al. 2009). Endosperms of GM wheat (EM12) showed light yellow colour and produced 10.8-fold (4.96 μg/g) more carotenoid content than non-transgenic wheat (EM12). In

another biofortification event, bacterial *CrtB* and *CrtI* genes were transformed into the Bobwhite wheat cultivar (Wang et al. 2014). The GM wheat thus produced contained darker red/yellow grains with around eightfold (4.76 μg/g) increased carotenoid content than non-transgenic wheat control. In GM Bobwhite wheat, the provitamin A content (commutative of α-carotene, β-carotene, and β-cryptoxanthin) increased 76-fold (3.82 μg/g) in the seed dry weight (Wang et al. 2014).

Iron biofortification has been attempted in wheat using *ferritin*, a popularly used gene for this purpose. The soybean *ferritin* gene, expressed under the ubiquitin-1 (maize) constitutive promoter, accumulated 40 μg/g of iron in the wheat leaves (Drakakaki et al. 2000). Overexpressing of *ferritin* (*TaFer1-A*) gene under a seed-specific promoter, resulted in 50–85% (up to 44.5 μg/g) increased iron accumulation in the endosperm of biofortified wheat (Borg et al. 2012).

4 Maize Biofortification

Transgenic approaches have been successfully implemented to develop provitamin A biofortified maize. The bacterial *crtB* and *crtI* genes, under an endosperm-specific 'super gamma-zein' promoter, were expressed in maize. The GM maize produced up to 34-fold (9.8 μg/g) more β-carotene in the endosperm than non-transgenic control maize (Aluru et al. 2008).

To develop β-carotene biofortified maize, the corn *psy1* and the bacterial (*E. uredovora*) *crtI* genes were introduced in an elite white corn variety M37W. The *psy1* gene was expressed under the wheat LMW glutenin promoter, and *crtI* gene was expressed under the barley D-hordein promoter (Naqvi et al. 2009). In this biofortified maize line, the endosperm accumulated around 60 μg/g β-carotene, 23 μg/g lycopene, and 36 μg/g zeaxanthin. Biofortified maize could contribute to reducing health problems due to VAD, in the future.

5 Cassava Biofortification

Cassava (*M. esculenta*) is the most important tropical root crop serving as a source of calories for more than 600 million Africans, Asians, and Latin Americans (http://www.fao.org/english/newsroom/news/2002/10541-en.html, accessed on 20 August 2020). Difficult, unwieldy breeding cycles, and limited knowledge of this starchy root, demands molecular breeding approaches for its improvement. As roots of commercial cassava cultivars contain limited provitamin A carotenoids, biofortification would be a promising approach for the nutritional improvement of this crop. The bacterial *crtB* gene was transformed to produce carotenoids in cassava. The transgenic cassava roots were yellow-fleshed with high carotenoid accumulated of up to 6.67 μg/g (Welsch et al. 2010).

6 Synergistic Effect

As discussed, iron biofortification also increases zinc accumulation in (rice) seeds. There is a natural synergistic relationship between iron, zinc, and carotenoids which facilitates their accumulation in plants and their bioavailability in humans. Synergy in the absorption of iron and zinc in plant cells has been established (King et al. 2000). Reports showed that iron and zinc bioavailability increased in the human gut due to carotenoids (Christian and West Jr 1998; Graham and Rosser 2000). A study in wheat showed a relationship between the plant protein content and accumulation of iron and zinc in seeds (Ozturk et al. 2006). The *Gpc-B1* (grain protein content) is one such gene that has been identified in wheat which increased protein along with zinc and iron content in the grain (Uauy et al. 2006). More knowledge in such synergistic relationships can help to design better biofortification strategies in the future.

7 Available Biofortified Crops

Biofortified crops developed through conventional breeding have been released and tested in many countries. ICAR-IIRR, India, developed zinc biofortified rice variety DRR Dhan 45 which accumulated zinc up to 24 μg/g in polished grain (https://icar.org.in/node/6293, accessed on 30 August 2020). DRR Dhan 45 is of good cooking quality (20.7% amylose content) with abiotic stress resistance to rice blast disease (*Magnaporthe grisea*), sheath rot disease (*Sarocladium oryzae*), and rice tungro virus infection.

CGIAR-HarvestPlus, the famous biofortification project, is involved in the testing and release of more than 300 varieties of 11 staple crops in more than 60 countries (https://www.harvestplus.org, accessed on 30 August 2020) (Fig. 17.6a). In India, high iron pearl millet (varieties ICTP 8203-Fe-10-2, ICMH 1201) and zinc-rich wheat (varieties BHU-3, BHU-6) have been developed by HarvestPlus in partnership with ICRISAT and CIMMYT (https://www.harvestplus.org/where-we-work/india, accessed on 30 August 2020). The CGIAR-HarvestPlus released a zinc biofortified rice variety in Bangladesh in 2013, and about 1.5 million farming households accepted and have since been growing them (Goldstein 2018). Some other zinc-rich rice varieties (BRRI dhan62, BRRI dhan72, BRRI dhan64) have been developed in Bangladesh by HarvestPlus in partnership with BRRI, IRRI, and BSMRAU (https://www.harvestplus.org/where-we-work/bangladesh, accessed on 30 August 2020). Provitamin A biofortified cassava (varieties NR 07/0220, UMUCASS 44; TMS 07/0593, UMUCASS 45; TMS 07/539, UMUCASS 46; etc.) and maize (varieties like Oba Super 6, Ife Hybrid 4, Sammaz 37, 38 and 39; orange maize, SC 510, SDM4, PVA 2) have been developed in Nigeria by HarvestPlus in partnership with NRCRI and IITA (https://www.harvestplus.org/where-we-work/nigeria, accessed on 30 August 2020). Available iron biofortified pearl millet and beans can provide our daily iron needs up to 80%. Zinc biofortified rice, wheat, and maize can provide our daily zinc needs up to

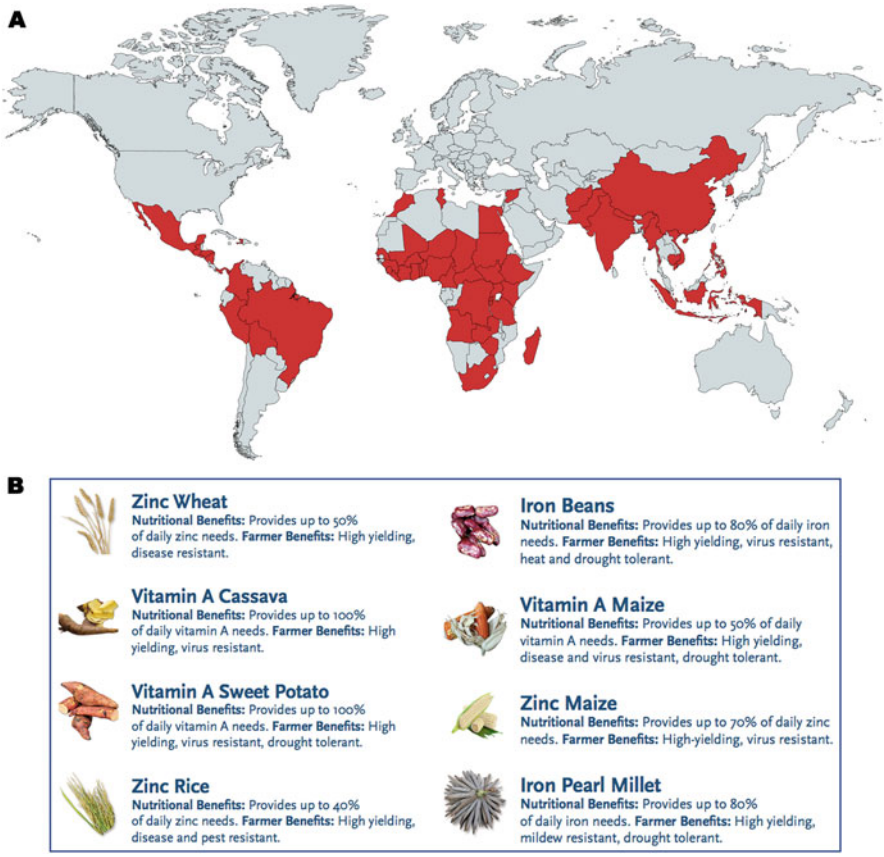

Fig. 17.6 Available biofortified crops in the world. (**a**) Countries where biofortified crops have been released and tested. (**b**) Available biofortified crop types in the world developed through conventional breeding. This infographic is created based on information from https://www.harvestplus.org/, accessed on 30 August 2020

40%, 50%, and 70%, respectively. Provitamin A biofortified cassava, maize, and sweet potato can provide our daily vitamin A needs up to 100%, 50%, and 100%, respectively (Fig. 17.6b).

8 Challenges in Transgenic Biofortification Crops

Conventional biofortification has been achieved through breeding of crops that are nutritionally rich and high yielding. Modern breeding techniques like marker-assisted breeding, genomics-assisted breeding, genome-wide association studies (GWAS), QTLs (quantitative trait locus), and SNP (single-nucleotide polymorphism) markers have been applied to develop such biofortified plant varieties.

Conventional breeding can assist in the varietal improvement, but it is limited and based on the available natural gene pool within a species. Sometimes, it is challenging to find a nutritionally rich variety from the available plant biodiversity. More than 20,000 cultivars of rice from Asia, Latin America, and the Caribbean have been screened for high iron and zinc, and nothing has been found as a promising variety for biofortification projects (Slamet-Loedin et al. 2015). This limitation of inferior gene pool in a crop species or species barrier can be easily solved by molecular breeding (GM approach), and rapid biofortification of crops can be achieved. Many biofortified crops have been developed by a transgenic approach as we have discussed in this chapter. Some of these GM biofortified crops like golden rice have been tested for the last two decades before being available for cultivation. Three renowned international food safety regulatory agencies: Food Standards Australia New Zealand, Health Canada, and the US Food and Drug Administration found golden rice safe for humans and recommended commercialization (Majumder et al. 2019). Unfortunately, going through the regulatory process for GM biofortified crops is a major challenge, especially in Asian, African, and other developing countries where it is needed the most. Most of these countries either do not have proper guidelines or they have a time-consuming regulatory process or lack political will. GM biofortified crop regulatory agencies of the developing countries do not have sophisticated laboratories for food quality and safety analysis, necessary infrastructure, and marketing strategies. Many of these countries have not yet decided the fate of CRISPR/Cas9 crops. The USDA has exempted the CRISPR-edited crops from the GM regulations, whereas the Court of Justice of the European Union has included it under GMO regulation guidelines.

Biofortified crops developed by private companies also demand some legal formalities before implementation in any country-specific GM regulation process. In such cases, better policies on public-private partnership (PPP model), freedom to operate (FTO), international crops distribution policies and awareness on health benefits of biofortified crops could expedite the process.

9 Conclusion

Nutritional security, along with food security, is the need of the hour to feed the increasing population. Biofortified crops can help to achieve this goal of achieving global nutritional security. Provitamin A, high iron, and high zinc biofortified crops have the potential to fight against VAD, IDA, and zinc deficiency and related health problems in men, women, and children. Biofortified crops that are developed by conventional breeding have established their potential and importance in selected countries. Now is the time to speed up and expand the process of staple crops biofortification involving more crops with the help of molecular breeding tools like genetic engineering and the CRISPR-Cas9 technology. Delay in the process means more lives are being threatened by hidden hunger which directly affects the people and the economy of a country.

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Agronomic and Transgenic Approaches for Rice Zn Biofortification

18

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Abstract

In humans, zinc deficiency can affect a large number of populations and may cause decreased height, diarrhea, and pneumonia, and also result in higher infant mortality rates. Zn deficiency may appear due to the food produced in ïnfertile lands and also due to the consumption of low Zn foods. Cereal-based diets such as rice (*O. sativa* L.) are more likely to have micronutrient deficiencies. Present understanding shows that achieving high levels of Zn in rice grains will need a combination of complementary approaches, including transgenic, agronomic practices and breeding. To do that, we should have a deep understanding about Zn uptake, distribution within the plant and loading in developing seeds. This chapter covers the latest updates about the agronomic and transgenic approaches for Zn biofortification in rice which is one of the most important staple food crops of the world.

Keywords

Foliar spray · *O. sativa* · Zinc homeostasis · Zn fertilization · Zn transporters

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1 Introduction

Inadequate consumption of key micronutrients, also known as "hidden hunger," is a serious public health problem affecting more than two billion people around the world (Abeshu and Geleta 2016). Micronutrient deficiency negatively affects key processes of physical and mental development and in extreme cases can lead to disease susceptibility, mental retardation, and blindness (Pinkaew et al. 2013). Iron (Fe) and zinc (Zn) deficiency are the most common mineral deficiencies, and Zn deficiency is considered more difficult to diagnose (Graham et al. 2012). Zn deficiency affects, approximately, 17.3% of the world's population (Wessells and Brown 2012), and 116.000 deaths of children aged 1–5 years are caused a year (Black et al. 2013). Zn deficiency can cause growth problems, such as decreased height, diarrhea, and pneumonia, and contribute to higher infant mortality rates (White and Broadley 2011).

The Zn deficiency disorder in humans arises as a result of food production in infertile lands and consumption of low Zn foods (Cakmak 2008; Cakmak and Kutman 2018). Cereal-based diets such as rice (*O. sativa*) are more likely to have micronutrient deficiencies (Gomez-Galera et al. 2010). Although rice is a staple food for over half of the world's population, especially in developing countries (Muthayya et al. 2014), rice grains have low concentrations of minerals such as Zn and iron (Fe) (Impa et al. 2013; Garcia-Oliveira et al. 2018). The polished grains of most rice varieties contain low amounts of these nutrients, providing only 20% of the recommended daily dietary requirement for Zn (Johnson et al. 2011; Lu et al. 2013). In fact, rice has the lowest concentration for both micronutrients and the narrowest genetic variability among the most commonly cultivated cereals (Garcia-Oliveira et al. 2018). Approximately 50% of cultivated soils have insufficient Zn, which can exacerbate the problem of low Zn concentration in rice (Cakmak 2008).

Increasing nutrient content in edible plant organs using biofortification, which may be accomplished by breeding, genetic engineering, and agronomic strategies, has been an economically viable alternative to combat micronutrient malnutrition (White and Broadley 2011; Phattarakul et al. 2012; Sperotto et al. 2012; Murgia et al. 2013; Ricachenevsky et al. 2015). Because rice has low concentrations of minerals and is widely consumed around the world, it is one of the main crops targeted for biofortification with Zn (Bouis and Saltzman 2017).

Grain Zn concentration is influenced by internal plant factors such as root Zn uptake, translocation, internal remobilization for developing organs (including seeds), and external factors such as Zn supply and soil conditions (Wissuwa et al. 2008; Bashir et al. 2012). Zn can be transported to rice grain by remobilization from leaves and other organs or by soil uptake after flowering (Sperotto 2013; Stomph et al. 2014). Several transporters related to Zn homeostasis have been characterized in rice; however, little is known about how Zn is transported from leaves to the seeds (Olsen and Palmgren 2014; Ricachenevsky et al. 2015). To increase the Zn concentration in grains, it is necessary to understand how rice uptake distributes and stores Zn in its tissues and which proteins are involved in each process. Therefore, rice

transporters involved in Zn movement within the plants and to grains still need to be better characterized.

This chapter presents the known genes involved in the control of Zn concentration in rice seeds, and the genetic engineering attempts performed to increase Zn abundance in grains. We also briefly review the agronomic practices that have shown to increase seed Zn concentration, which can be combined with the best available genetic materials for biofortification purposes.

2 Genes Controlling Zn Concentrations in Rice

Zinc homeostasis in rice has been widely studied in the last years (for a comprehensive review, see Ricachenevsky et al. 2015). Therefore, our goal here is to present the molecular players that were shown to regulate Zn concentration in rice seeds, whether or not they have been used in biofortification efforts. Table 18.1 lists all the genes discussed in this section.

The literature is scarce about genes that control Zn (and Fe) levels in rice seeds (Whitt et al. 2018). Genes from the ZIP (Zinc-regulated/Iron-regulated transporter Protein) family were implicated in Zn transport in rice plants. OsZIP4, OsZIP5, and OsZIP8 are plasma membrane Zn transporters upregulated under Zn deficiency in both shoots and roots. Overexpression of each gene results in decreased Zn concentration in seeds and leaves and Zn accumulation in roots (Ishimaru et al. 2005, 2007; Lee et al. 2010a, b). Still, their precise role in Zn homeostasis is unknown.

Recently, a series of studies have shown the importance of OsZIP9 to Zn uptake from the soil into rice plants, with consistent results (Huang et al. 2020; Tan et al. 2020; Yang et al. 2020b). OsZIP9 is expressed in root epidermis and exodermis, is upregulated by Zn deficiency, is located at the plasma membrane, and shows high efficiency in Zn transport. Knockout *oszip9* plants show Zn-deficient phenotype and had decreased overall Zn uptake, with decreased Zn concentrations in roots, shoots, and seeds (Huang et al. 2020; Tan et al. 2020; Yang et al. 2020b). Interestingly, *OsZIP9* was shown to be partially redundant with *OsZIP5*, which is located *in tandem* with *OsZIP9* in chromosome 5. Double mutants *oszip5oszip9* present even more pronounced Zn-deficient phenotype. *OsZIP9* is regulated by systemic Zn deficiency signaling, whereas *OsZIP5* is upregulated in roots by local Zn deficiency (Tan et al. 2020). Moreover, increased *OsZIP9* expression was associated with high Zn concentration in seeds of selected rice genotypes, suggesting that OsZIP9 might be linked to rice natural variation, and therefore may be used in breeding Zn-biofortified plants (Yang et al. 2020b). These studies establish the central role of OsZIP9 in rice Zn uptake.

OsZIP1, which was thought as being important for Zn uptake in roots, was suggested to be involved in Zn detoxification. OsZIP1 is localized to plasma membrane and endoplasmic reticulum and is induced by high Zn concentrations, and knockout *oszip1* plants have increased Zn (and other metals) concentration in roots upon Zn excess. *oszip1* plants are also more sensitive to high Zn supply, suggesting that OsZIP1 probably detoxifies Zn (Liu et al. 2019).

Table 18.1 Genes identified as affecting Zn concentration in rice plants

Gene	Locus MSU	Locus RAP-DB	Subcellular localization	Expression	Regulation	Changes in Zn concentration[a]	References
OsZIP1	LOC_Os01g74110	Os01g0972200	Plasma membrane, endoplasmic reticulum	Roots	Up in roots under Zn excess	Zn increase under Zn excess, Zn decrease under deficiency, both in roots	Liu et al. (2019)
OsZIP3	LOC_Os04g52310	Os04g0613000	Plasma membrane	Shoot basal region, nodes	No	Zn increase in old leaves, decrease in developing tissues	Sasaki et al. (2015)
OsZIP4	LOC_Os08g10630	Os08g0207500	Plasma membrane	Vascular bundles, especially in phloem cells leaves, stem and roots; root and shoot apical meristem	Up in shoots and roots under Zn deficiency	*No mutant available*	Ishimaru et al. (2005)
OsZIP5	LOC_Os05g39560	Os05g0472700	Plasma membrane	Root epidermis and stele, xylem parenchyma cells of enlarged vascular bundles, and phloem and xylem of diffuse vascular bundles in the rice node	Up in shoots and roots under Zn deficiency	Zn decrease in roots, shoot basal region, leaves	Lee et al. (2010a); Tan et al. (2020)
OsZIP7	LOC_Os05g10940	Os05g0198400	Plasma membrane	Parenchyma cells of enlarged vascular bundles in nodes, stele in roots	Up in shoots and roots under Zn deficiency	Zn increase in roots and shoot basal region, decrease in leaves and seeds	Ricachenevsky et al. (2018); Tan et al. (2019); Gindri et al. (2020)

						Similar changes for Cd	
OsZIP8	LOC_Os07g12890	Os07g0232800	Plasma membrane	Shoots and roots	Up in shoots and roots under Zn deficiency	*No mutant available*	Lee et al. (2010b)
OsZIP9	LOC_Os05g39540	Os05g0472400	Plasma membrane	Exodermis and endodermis of the root mature region (Huang et al. 2020); Root epidermis, xylem parenchyma cells of enlarged vascular bundles, and phloem and xylem of diffuse vascular bundles in the rice node (Tan et al. 2020); epidermis and exodermis of lateral roots (Tan et al. 2020)	Up in shoots and roots under Zn deficiency	Zn decrease in roots, shoot basal region, leaves	Huang et al. (2020); Tan et al. (2020); Yang et al. (2020b)
OsHMA2	LOC_Os06g48720	Os06g0700700	Plasma membrane	Pericycle of roots, vascular bundles of nodes	Not affected or slightly decreased by Zn deficiency in roots	Zn decrease concentration in upper nodes and reproductive tissues, increase in roots	Takahashi et al. (2012); Satoh-Nagasawa et al. (2012); Yamaji et al. (2013)

(continued)

Table 18.1 (continued)

Gene	Locus MSU	Locus RAP-DB	Subcellular localization	Expression	Regulation	Changes in Zn concentration[a]	References
OsVMT/ OsZIFL12	LOC_Os12g03899	Os12g0133100	Vacuole	Exodermis, endodermis; parenchyma cell bridge, xylem region of enlarged vascular bundles and diffuse vascular bundles of nodes	Up in roots under Fe deficiency	Zn decrease in seeds	Che et al. (2019)
OsVIT1	LOC_Os04g38940	Os04g0463400	Vacuole	Mainly flag leaves, roots	Up in roots and shoots under Fe excess	Zn increase in seeds (mainly embryo) and decrease in flag leaves	Zhang et al. (2012)
OsVIT2	LOC_Os09g23300	Os09g0396900	Vacuole	Mainly flag leaves, roots	Not affected	Zn increase in seeds (mainly embryo) and decrease in flag leaves	Zhang et al. (2012)

[a]Changes caused by knockout/knockdown. Effects of overexpression are not included

OsZIP7 was shown to be also a plasma membrane Zn transporter upregulated by Zn deficiency, which can alter Zn partitioning when expressed constitutively in *A. thaliana*. These plants showed increased Zn concentrations in shoots and seeds, whereas concentration in roots was decreased (Ricachenevsky et al. 2018). Conversely, loss-of-function plants for OsZIP7 show decreased shoot and seed Zn concentration, suggesting OsZIP7 is involved in root-to-shoot Zn translocation, and Zn node intervascular transfer to developing grains (Ricachenevsky et al. 2018; Tan et al. 2019; Gindri et al. 2020). These results indicate that OsZIP7 might also be a good candidate for biofortification.

Interestingly, the node in rice is a hub for nutrient distribution in rice shoots, including developing leaves and panicles. Therefore, many transporters involved in transferring nutrients and trace elements between the different tissues in the complex vascular systems in the node have been characterized, which can affect the seed ionome (Yamaji and Ma 2014, 2017). Among these, OsZIP3 is highly expressed in basal and upper nodes of rice plants and is localized at the xylem-intervening parenchyma cells and xylem transfer cells of the enlarged vascular bundle. Knockdown *oszip3* plants showed decreased Zn concentration in the shoot basal region and elongating zone, but higher concentration in the xylem sap, at the vegetative stage. At the reproductive stage, leaves show increased Zn concentration, while nodes show decreased Zn concentration. These results support that OsZIP3 is necessary for proper Zn transport from nodes to developing tissues, which may include the seeds (Sasaki et al. 2015).

The OsHMA2 (Heavy Metal Associated) protein is a Zn and Cd plasma membrane transporter expressed in pericycle and nodes. There is still some controversy over the influx/efflux activity of OsHMA2, since different studies presented distinct results in yeast complementation assays (Satoh-Nagasawa et al. 2012; Takahashi et al. 2012; Yamaji et al. 2013). For its *in planta* role, OsHMA2 was first suggested to be involved in root-to-shoot translocation of both Zn and Cd by loading both elements in the xylem (Satoh-Nagasawa et al. 2012; Takahashi et al. 2012). Later, another work suggested that OsHMA2 is necessary for Zn delivery to developing tissues. Expression of OsHMA2 in pericycle cells would facilitate Zn transfer through the phloem to tissues that have high Zn requirement but no xylem transport, such as root and shoots meristems. OsHMA2 is also localized in the phloem region of the enlarged and diffuse vascular bundles of the nodes, and its loss-of-function results in decreased Zn concentration in reproductive organs (Yamaji et al. 2013). Although it is clear that OsHMA2 is key for proper Zn accumulation in rice seeds, it remains to be explored if OsHMA2 also has a role in Zn transfer into developing seeds from mother-plant tissues, as demonstrated for its *A. thaliana* orthologs AtHMA2 and AtHMA4 (Olsen et al. 2016).

Another transporter expressed in the nodes and regulating Zn concentration in rice seeds is the recently characterized OsVMT (Vacuolar Mugineic acid Transporter)/OsZIFL12 (ZINC-INDUCED FACILITATOR-LIKE) (Ricachenevsky et al. 2011; Che et al. 2019). OsVMT/OsZIFL12 is part of the ZIFL family of transporters, which include transporters of nicotianamine and phytosiderophore in plants, such as AtZIF1 (Haydon et al. 2012) and OsTOM1/OsZIFL4 (Nozoye et al.

2011; Ricachenevsky et al. 2011). Results showed that OsVMT/OsZIFL12 is a vacuolar DMA (deoxumugineic acid, the major phytosiderophore secreted by rice plants to acquire Fe^{3+} from the soil) transporter involved in Fe storage in root vacuoles. OsVMT/OsZIFL12 is also highly expressed in the node, especially in the parenchyma cell bridge, a tissue where Fe and Zn accumulate (Moore et al. 2014; Zhao et al. 2014; Yamaji and Ma 2019). DMA chelates Zn as well (Suzuki et al. 2008), and loss-of-function of OsVMT increases both Zn and Fe concentration in seeds, suggesting that lack of DMA to chelate metals in the vacuole of node tissues increases their availability in the cytosol, increasing their translocation and accumulation in seeds. This is supported by the finding that DMA is also increased in seeds of *osvmt* mutants. Interestingly, accumulation of Zn and Fe was increased in the endosperm, indicating that Zn-DMA and Fe-DMA complexes may be easier to load in the inner tissues of seeds (Che et al. 2019).

Rice genome has two VIT (Vacuolar Iron Transporter) genes, named *OsVIT1* and *OsVIT2*. Distinctly from the *A. thaliana* ortholog, AtVIT1, which transports Fe and manganese (Mn), but not Zn (Kim et al. 2006), OsVIT1 and OsVIT2 are able to transport Zn into the vacuole. Both are highly expressed in flag leaves, and since single mutants for each gene result in increased levels of Zn (and Fe) in seeds, especially in embryos, it was proposed that the absence of vacuolar storage in flag leaves would increase Zn availability for phloem translocation to developing seeds. Therefore, the model implies that OsVIT1 and OsVIT2 have an indirect role in controlling Zn concentration and localization in seeds (Zhang et al. 2012). However, Fe localization was shown to be altered in seeds of mutant plants, leaving an open question as to whether Zn localization is directly controlled by OsVIT1 and OsVIT2.

3 Transgenic Strategies for Increasing Zn in Rice Grains

Biofortified plants have been developed by genetic modification over the last 20 years. Crops that have been biofortified using transgenics include rice (*O. sativa*), cassava (*Manihot esculenta*), maize (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and potatoes (*Solanum tuberosum*), while micronutrients increased using biotechnology include mainly Fe, Zn, and vitamin A, as well as a reduction in antinutritional factors, such as cyanogens and phytates (Hefferon 2019). Even though Zn accumulation in rice seeds is a complex and not yet fully understood process, considerable progress has been made in recent years to modify seed Zn content via genetic engineering through overexpression of different Zn-related proteins (Sperotto et al. 2018), mainly involved with three different mechanisms: (1) Zn uptake from the soil by the roots; (2) Zn transport and distribution within the aerial parts of the rice plant; and (3) Zn import and accumulation in rice seeds. It is important to highlight that Zn and Fe are likely to share at least some of the transporters, chelators, and translocation machinery that control these processes (Kawakami and Bhullar 2018), and therefore addressing Zn deficiency or biofortification in rice can also result in an increased accumulation of Fe (Hefferon 2019). In fact, most of the transgenic strategies that resulted in increased Zn

concentration in rice seeds were primarily focused on increasing Fe concentration. Thus, concomitant Zn and Fe biofortification in rice seeds might be achieved by the same transgenic approach (Sperotto et al. 2012).

Rice tends to be naturally low in Zn and Fe (Garcia-Oliveira et al. 2018). The low Zn concentration is further exacerbated due to processing, which removes the nutrient-rich outer layers of the embryo (Hefferon 2019). In fact, rice has the lowest baseline Zn and Fe concentrations among cereals (Garcia-Oliveira et al. 2018). For this reason, most of the strategies have focused on the increased Zn concentration in polished seeds (white rice; endosperm). Nutritional studies suggested that 28 mg/kg Zn concentration in white grain is essential to reach the 30% of human estimated average requirement (Bouis et al. 2011; Slamet-Loedin et al. 2015; Kawakami and Bhullar 2018). Considering that the rice genetic variation in Zn concentration extend somewhat above this target, ranging from 16 to 58 mg/kg in unpolished seeds (brown rice), but only 8 to 16 mg/kg in polished seeds (white rice), rice germplasm diversity has been exploited to breed Zn-dense varieties conventionally, reaching up to 24 mg/kg Zn in white rice (Bouis et al. 2011; Slamet-Loedin et al. 2015; Kawakami and Bhullar 2018). Even though breeding efforts for high Zn have been partially successful in rice (Sanjeeva Rao et al. 2020), it seems that most of the conventional breeding approaches alone might not be able to increase the Zn content to the final target value set by the HarvestPlus program (28 mg/kg Zn in white grain - Bouis et al. 2011; Vasconcelos et al. 2017).

On the other hand, using genetic modification, several groups using different strategies were able to achieve the target level. In Table 18.2, we present all transgene-using trials aiming at rice Zn biofortification. Fifteen out of the 17 studies that analyzed Zn concentration in polished rice seeds presented at least 28 mg/kg Zn in white grain, with the highest value (76 mg/kg Zn) found by Johnson et al. (2011), which constitutively overexpressed the rice *nicotianamine synthase 2* (*OsNAS2*) gene in cv. Nipponbare plants, obtaining 2.2-fold higher concentration than the wild-type. Similar increase was also detected in brown/unpolished rice, reaching up to 91 mg/kg Zn. We also highlight the successful strategy of Banakar et al. (2017), which constitutively overexpressed the rice *nicotianamine synthase 1* (*OsNAS1*) and the barley *nicotianamine aminotransferase* (*HvNAATb*) genes using the maize *Ubiquitin* promoter, reaching up to 65 mg/kg Zn in white rice (4.1-fold higher than the wild-type cv. EYI 105). Both strategies (Johnson et al. 2011; Banakar et al. 2017) focused on increasing nicotianamine (NA)/phytosiderophore (PS) levels, which are responsible for Zn (and Fe) transport and distribution within the aerial parts of the rice plant.

In order to further analyze these results, we considered as average reference daily intakes (RDI) the range of 8–11 mg of Zn (Gomez-Galera et al. 2010); USDA—https://www.nal.usda.gov/fnic/zinc/). Considering this RDI, we calculated the percentage of Zn RDI that a meal containing 100 g of dry rice would contribute with, for brown rice and white rice (when available) from each report (Table 18.2). Considering brown rice (unpolished seeds), only 4 out of the 20 approaches shown in Table 18.2 have fulfilled 100% of the lowest Zn RDI level (8 mg): (1) constitutive overexpression of *OsNAS2* gene (Johnson et al. 2011); (2) a rice mutant obtained by

Table 18.2 Seed Zn concentration of transgenic plants and an estimate of dietary minimum Reference Daily Intake (RDI) fulfilled per meal (100 g of dry seeds)

Background cultivar	Gene	Tissue (promoter)	Seed Zn concentration[a] (fold-change)	% of RDI (100 g of dry seed)[b]	References
Japonica cv. Tsukinohikari	Barley *IDS3*	Root (native promoter)	15 in unpolished seeds (1.4-fold increase)	14–19% (unpolished seeds)	Suzuki et al. (2008)
Japonica cv. Nipponbare	*OsNAS1*	Constitutive (2X*CaMV 35S*)	63 in unpolished seeds (1.5-fold increase)/48 in polished seeds (1.4-fold increase)	57–79% (unpolished seeds)/ 44–60% (polished seeds)	Johnson et al. (2011)
	OsNAS2		91 in unpolished seeds (2.2-fold increase)/76 in polished seeds (2.2-fold increase)	83–114% (unpolished seeds)/ 69–95% (polished seeds)	
	OsNAS3		65 in unpolished seeds (1.5-fold increase)/49 in polished seeds (1.4-fold increase)	59–81% (unpolished seeds)/ 45–61% (polished seeds)	
Japonica cv. Kitaake	*OsNAS2* (activation tagging)	Constitutive (maize *ubiquitin*)	63 in unpolished seeds (3.2-fold increase)/45 in polished seeds (2.9-fold increase) [c]	57–79% (unpolished seeds)/ 41–56% (polished seeds)	Lee et al. (2011)
Japonica cv. Tsukinohikari	*OsTOM1*(*TRANSPORTER OF MUGINEIC ACID 1*)	Constitutive (*CaMV 35S*)	47 in unpolished seeds (1.7-fold increase) [c]	43–59% (unpolished seeds)	Nozoye et al. (2011)
Japonica cv. Dongjin	*OsVIT1* and *OsVIT2* (T-DNA insertion mutant lines)	–	51 in unpolished seeds (1.5-fold increase) [c]	46–64% (unpolished seeds)	Zhang et al. (2012)
Myanmar rice tropical *Japonica* cv. Paw San Yin	Soybean *ferritin* + barley *NAS1* + *OsYSL2*	Endosperm (*OsGlb-1* and *OsGluB-1*) + constitutive (*OsActin1*) + endosperm (*OsSUT1* and *OsGlb-1*)	52 in unpolished seeds (1.2-fold increase)/39 in polished seeds (1.2-fold increase)[c]	47–65% (unpolished seeds)/ 35–49% (polished seeds)	Aung et al. (2013)

Japonica cv. unknown	*Malus xiaojinensis IRT1*	Constitutive (*CaMV 35S*)	45 in unpolished seeds (3.0-fold increase)	41–56% (unpolished seeds)	Tan et al. (2015)
Japonica cv. Taipei 309	Arabidopsis *IRT1* + Arabidopsis *NAS1* + bean *ferritin*	Vascular tissue and root epidermal cells (*Medicago sativa EARLY NODULIN 12B—ENOD12B*)	43 in unpolished seeds (1.5-fold increase)/33 in polished seeds (1.4-fold increase)[c]	39–54% (unpolished seeds)/ 30–41% (polished seeds)	Boonyaves et al. (2016)
Indica cv. IR64	Soybean *ferritin1* + *OsNAS2*	Endosperm (*OsGluA2*) + constitutive (*CaMV 35S*)	53 in polished seeds (3.8-fold increase)[c]	48–66% (polished seeds)	Trijatmiko et al. (2016)
Japonica cv. EYI 105	*OsNAS1* + barley *NAATb*	Constitutive (maize *ubiquitin*)	78 in unpolished seeds (4.2-fold increase)/65 in polished seeds (4.1-fold increase)	71–98% (unpolished seeds)/ 59–81% (polished seeds)	Banakar et al. (2017)
Japonica cv. Nipponbare	Arabidopsis *IRT1* + Arabidopsis *NAS1* + bean *ferritin*	Constitutive (native *pIRT1*) + constitutive (*CaMV 35S*) + Endosperm (*OsGlb-1*)	33 in polished seeds (1.8-fold increase)	30–41% (polished seeds)	Boonyaves et al. (2017)
Japonica cv. Nipponbare	Arabidopsis *NAS1* + bean *ferritin* + *Pantoea ananatis CRTI* (*CAROTENEDESATURASE*) + maize *PSY* (*PHYTOENE SYNTHASE*)	Constitutive (*CaMV 35S*) + Endosperm (*OsGlb-1*) + Endosperm (*OsGluB-1*) + Endosperm (*OsGluB-1*)	30 in polished seeds (1.3-fold increase)	2–38% (polished seeds)	Singh et al. (2017)
Japonica cv. Dong bei xiang	Unknown (mutant obtained by γ ray treatment)	–	Field conditions: 53 in unpolished seeds (2.2-fold increase) Lab conditions (nutrient solution with 50 μM Zn): 105 in unpolished seeds (1.4-fold increase)[c]	Field conditions: 48–66% (unpolished seeds) Lab conditions (nutrient solution with 50 μM Zn): 95–131% (unpolished seeds)	Wang et al. (2017)

(continued)

Table 18.2 (continued)

Background cultivar	Gene	Tissue (promoter)	Seed Zn concentration[a] (fold-change)	% of RDI (100 g of dry seed)[b]	References
Japonica cv. Kitaake	*OsHMA7* (*HEAVY METAL-TRANSPORTING P-TYPE ATPase 7*)	Constitutive (maize *ubiquitin*)	90 in unpolished seeds (2.7-fold increase)	82–112% (unpolished seeds)	Kappara et al. (2018)
Japonica cv. Nipponbare	Arabidopsis *FRD3* + Arabidopsis *NAS1* + bean *ferritin*	Constitutive (maize *ubiquitin*) + Constitutive (*CaMV 35S*) + Endosperm (*OsGlb-1*)	65 in unpolished seeds (2.4-fold increase)/40 in polished seeds (2.5-fold increase)	59–81% (unpolished seeds)/ 36–50% (polished seeds)	Wu et al. (2018)
Japonica cv. Nipponbare	Knockout of *OsVMT* (*VACUOLAR MUGINEIC ACID TRANSPORTER*) by CRISPR/Cas9	–	35 in polished seeds (1.6-fold increase)	32–44% (polished seeds)	Che et al. (2019)
Japonica cv. Nipponbare	Wheat *CNR5* (*CELL NUMBER REGULATOR 5*)	Constitutive (*CaMV 35S*)	84 in unpolished seeds (1.7-fold increase)[c]	76–105% (unpolished seeds)	Qiao et al. (2019)
Japonica cv. Nipponbare and *indica* cv. IR64	Arabidopsis *NRAMP3* + Arabidopsis *NAS1* + bean *ferritin*	Embryo/aleurone (rice *Oleosin 18*) + Constitutive (*CaMV 35S*) + Endosperm (*OsGlb-1*)	Nipponbare: 65 in unpolished seeds (2.2-fold increase)/46 in polished seeds (2.3-fold increase)[c] IR64: 49 in unpolished seeds (1.5-fold increase)/48 in polished seeds (2.7-fold increase)[c]	Nipponbare: 59–81% (unpolished seeds)/42–58% (polished seeds) IR64: 45–61% (unpolished seeds)/ 44–60% (polished seeds)	Wu et al. (2019)

[a]Expressed as μg Zn/g DW
[b]According to the minimum reference daily intakes (11–8 mg) (Gomez-Galera et al. 2010; USDA—https://www.nal.usda.gov/fnic/zinc/)
[c]Values estimated from figures presented on the cited references

γ ray treatment in an unknown gene, under lab conditions and with nutrient solution supplied with 50 μM Zn (Wang et al. 2017); (3) constitutive overexpression of *OsHMA7* (*heavy metal-transporting P-type ATPase*) gene (Kappara et al. 2018); and (4) constitutive overexpression of wheat *TaCNR5* (*cell number regulator*-5) gene (Qiao et al. 2019). Also, the constitutive overexpression of two genes (*OsNAS1* and barley *HvNAATb*) has fulfilled 98% of the lowest Zn RDI level (Banakar et al. 2017). When we consider the highest level (11 mg), no strategy has been successful in fulfilling 100% of the Zn RDI.

It is important to note that biofortification would only be effective when Zn concentration is increased in the rice endosperm, due to the removal of other parts during milling, leaving only the endosperm as the edible part (Matsuo et al. 1995; Sperotto et al. 2018). When we consider polished seeds (white rice), most of the transgenic strategies do not reach even 50% of the lowest Zn RDI level, while only two were able to fulfill more than 80%: constitutive overexpression of *OsNAS2* gene (Johnson et al. 2011), and constitutive overexpression of *OsNAS1* and *HvNAATb* genes (Banakar et al. 2017). Therefore, increased Zn accumulation in white rice is still a challenge for breeders and molecular biologists. We highlight that overexpression of several genes (involved with different Zn mechanisms) is not enough to guarantee an effective Zn accumulation in rice grains. As seen in Table 18.2, no strategy using three or more genes was able to generate rice plants with high Zn in polished seeds (Masuda et al. 2012; Aung et al. 2013; Boonyaves et al. 2016, 2017; Singh et al. 2017; Wu et al. 2018, 2019). Also, transgenic rice lines containing only one modified gene would probably be more easily accepted by consumers than lines containing numerous genes. This is also true for using self-genes (or at least from other plants) to produce biofortified rice lines, instead of using genes from bacteria or other organisms (Sperotto et al. 2018).

As previously pointed by Sperotto et al. (2018), it is important to reinforce that some aspects regarding Zn (or any other nutrient) biofortification are critical to find the best strategy, rice cultivar, and growth conditions. Therefore, the most effective strategies should be tested side by side using the following: (1) the same growth conditions (soil type influences the amount of available Zn—Vasconcelos et al. 2017); (2) the same background rice cultivar (Gregorio and Htut 2000), and preferably popular varieties possessing naturally high Zn, as well as high yield (in this case, Nipponbare would not be the best choice); (3) the same milling protocol, as the polishing time is critical for Zn concentration analysis (Sperotto et al. 2012); (4) the same Zn bioavailability tests (Johnson et al. 2011; Lee et al. 2011), which would require a better interaction between plant and human nutrition researchers; and (5) simultaneous analysis of other heavy metals or contaminants (such as Cd) that could be cotransported with Zn (Bashir et al. 2013; Slamet-Loedin et al. 2015; Trijatmiko et al. 2016; Banakar et al. 2017).

4 Zn Agronomic Biofortification

Different from transgenic biofortification (discussed in Sects. 2 and 3), which focus on developing rice genotypes that express the phenotype of high Zn concentration in seeds regardless of plant management, agronomic biofortification aims at identifying the optimal use of fertilizers and other management practices to increase Zn concentration in grains (Cakmak and Kutman 2018). An important rationale for combining both strategies is that Zn deficiency is a common problem, being one of the most widespread micronutrient disorders for rice (Wissuwa et al. 2006). Therefore, plants engineered for higher Zn loading in seeds might not express the desired phenotype due to low Zn availability in the soil (Cakmak and Kutman 2018). It is also relevant to note that Zn deficiency in humans overlaps worldwide with Zn-deficient soils (Cakmak 2008), making combined efforts in genetics and agronomic practices key to allow biofortified plants express their full potential where they are most needed.

Among the important factors for Zn agronomic biofortification is fertilization and growth conditions. Rice can be cultivated in either anaerobic, flooded conditions, or aerobic, unflooded soils (Gao et al. 2006; Pinson et al. 2015). From these, flooded conditions further increase likelihood of Zn deficiency for rice plants (Johnson-Beebout et al. 2009). Another factor for the effectiveness of agronomic practices is how Zn is applied: fertilization in the soil or by foliar Zn supplementation (Cakmak and Kutman 2018). Here we discuss how these factors can influence Zn agronomic biofortification.

Zn should only be applied when the content of native Zn in soils does not supply the demand of rice plants. Usually, the necessity for Zn application is established considering available Zn in soils, which can be estimated by different simple chemical extractors (e.g., HCl, Mehlich-1, EDTA, and others). Zn is found in the inorganic matter and in the mineral fraction, with lower content/concentrations, verified in the exchangeable fraction (i.e., bioavailable) and in the soil solution (Brunetto et al. 2018). Thus, for example, soil with higher levels of organic matter can have high Zn availability, and, consequently, less Zn application is needed. However, Zn availability depends on other variables, such as pH and cation exchange capacity (CEC) (Tiecher et al. 2017).

In soils cultivated with rice, some chemical fertilizers applied to supply N, P, and K may have Zn in their composition, which can be considered a contaminant. In addition, organic fertilizers have Zn in the composition. Also, Zn can enter areas cultivated with rice by irrigation water and fungicides, applied to control fungal diseases. The Zn export to the rice grain in general is small, contributing to the Zn cycling. All of this means that Zn application for plant growth is necessary in small doses.

Some studies report that Zn fertilization in either flooded or unflooded conditions results in little or no change in seed Zn concentration (Gao et al. 2006). A survey of 1763 worldwide rice cultivars showed that flooded and unflooded conditions result in similar average Zn concentration, suggesting that growth conditions have low impact on Zn loading in rice grains (Pinson et al. 2015). Another study using five rice cultivars and four different native soil types varying in Zn availability (from severely

deficient to high Zn) plus soil fertilization found that while fertilization has little effect on grain Zn (but change Zn levels in vegetative tissues), native Zn levels in soil were correlated with Zn found in seeds. Genotype variation was similar in all soils, except the severely Zn limited. Therefore, soils with high Zn and Zn-accumulating genotypes are two important factors for having high Zn levels in seeds, whereas fertilization is not that important. The authors conclude that while developing high Zn lines for specific environments might be feasible, genotypes that increase Zn levels in seeds in response to Zn soil fertilization seem more challenging (Wissuwa et al. 2008).

Other authors suggest that management practices, such as alternate wetting and drying, improves Zn fertilization for plant usage and could also increase Zn levels in grains (Johnson-Beebout et al. 2016). This is based on the observation that soil drying improves Zn availability, whereas submergence rapidly, and irreversibly, immobilize Zn applied as a fertilizer (Izquierdo et al. 2016). Indeed, one study support that view, showing that alternate wetting and drying increases Zn in grains by 9% on average compared to continuous flooding. Still, data suggest that management has a larger impact than soil fertilization with Zn (Tuyogon et al. 2016). Lastly, when comparing two cultivars with high Zn levels, it was found that both Zn fertilization and water management can have small but yet relevant impacts on Zn concentration. Yet, genotype seems to be the most relevant factor, as the cultivar showing continuous root uptake increased Zn levels to a higher degree, responding to drying and fertilization (Johnson-Beebout et al. 2016). This is in agreement with a model for Zn supply to grains proposed by (Sperotto 2013) and supported by evidence (Impa et al. 2013), which states that rice plants preferentially use primary root uptake as Zn source for grain filling if Zn supply is abundant, whereas Zn remobilization is preferentially used when Zn is limiting. Also, Zn mobility in the phloem can vary between genotypes (Impa et al. 2013). In conclusion, although fertilization and water management seem to increase Zn in rice grains in specific genotypes, natural soil Zn concentration and genotype seem to be the most relevant factors.

Zn foliar application can be carried out in rice plants. However, most of the time, the amount of Zn absorbed by the leaves is small, accumulated and redistributed to other organs. This happens especially for some factors, greater thickness of the cuticle in leaves, which can make it difficult to absorb the ion; washing of the ion on the leaf surface by rainwater, or even by other factors related to plants or climate, but also because the Zn concentrations in the applied solutions are small, to avoid damage to the leaf tissue. Therefore, leaf applications of Zn normally contribute little to the mineral nutrition of plants. However, they can contribute to increase Zn concentrations in grains.

Concerning foliar fertilization, one study including 17 field trials in 5 countries (China, India, Turkey, Thailand, and Lao PDR), 7 cultivars, soils with acid to basic pH, and 0.5–6.5 mg/kg extractable Zn, once again found that soil fertilization has no impact in seed Zn concentration, whereas foliar Zn application was more effective. On average, authors found 25–32% increase in Zn levels in seeds when foliar Zn was

applied, compared to only 2.4% when Zn was added to the soil. It is important to highlight that foliar Zn application should be performed after flowering, when the seeds are filling (Phattarakul et al. 2012). Similar results were found for wheat when comparing soil and foliar Zn application for biofortification (Cakmak and Kutman 2018). Studies also highlight that, besides the timing of foliar Zn application, genotypes respond differently to such fertilization, with those with high remobilization capacity increasing their Zn concentration in seeds to a larger extent (Mabesa et al. 2013). Interestingly, the increase in Zn concentration due to foliar Zn application seems to benefit seed germination, resulting in more vigorous seedlings (i.e., larger/longer roots and coleoptile) (Boonchuay et al. 2013).

Another important factor for Zn foliar fertilization aiming biofortification is the Zn form used. The most common used form is Zn sulfate ($ZnSO_4.7H_2O$) (Boonchuay et al. 2013; Mabesa et al. 2013). A comparison between Zn sulfate, Zn amino acid complex, Zn citrate, and Zn EDTA showed that Zn sulfate and Zn amino acid, when applied as foliar spray, increase 22–25% the Zn concentration in white rice, whereas Zn citrate and Zn-EDTA result in increases of only 10–13%. Interestingly, Zn sulfate and Zn amino acid also increase Zn bioavailability by 65–69%. This is likely a result of reduced levels of the antinutrient phytic acid when Zn sulfate and Zn amino acid are applied, since phytic acid reduces Zn availability for absorption. Both foliar Zn forms resulted in no changes in grain yield, showing that foliar Zn application can be used to generate biofortified grains without penalty in productivity (Wei et al. 2012).

Since Zn fertilization via both soil and foliar spray is influenced by several different factors, it is important to understand which other factors could increase fertilization effectiveness. It is suggested, for example, that nitrogen (N) soil fertilization combined with Zn could improve Zn in grains, whereas phosphate addition could slow down Zn uptake due to adsorption to soil particles, which suggest that fertilization for Zn biofortification might be combined with other common fertilization practices (Nakandalage et al. 2016). In this line, one large study showed that combination of soil and foliar Zn application resulted in maximum increase in seed Zn concentration along with decreased phytic acid levels and increased bioavailability. However, the same treatment resulted in decreased Fe concentration and showed to be, at least partially, a genotype-specific response (Saha et al. 2017), suggesting that some fine-tuning is necessary to achieve the best results. Comparable results were described in a recent study using 26 rice cultivars (Saha et al. 2020), showing that combination of soil and foliar fertilization might be the most effective solution for agronomic Zn biofortification.

5 Concluding Remarks and Future Directions

The current knowledge shows that achieving high levels of Zn in rice grains will require complementary approaches, including transgenic, agronomic practices, and breeding. To do that, we will need to deepen our understanding of Zn uptake, distribution within the plant, and loading in developing seeds. Despite the recent

discovery of OsZIP9 role in Zn uptake, likely working at least partially redundant with OsZIP5 (Huang et al. 2020; Tan et al. 2020; Yang et al. 2020b), we still have a blurry picture of Zn acquisition by rice roots. One possibility is that phytosiderophore secretion to the rhizosphere, Zn chelation, and Zn-phytosiderophore uptake might have a role (Nakandalage et al. 2016), although the precise contribution of each uptake system is not clear. The presence of such dual transport strategies would be analogous to the combined strategy found in rice and other *Oryza* genus species (Ricachenevsky and Sperotto 2014; Wairich et al. 2019; de Oliveira et al. 2020). However, evidences for such a system are lacking. It will also be important to fully understand how Zn is loaded in rice-developing seeds, including the role of ZIPs and HMAs, and the contribution of DMA and NA to Zn translocation to developing seeds. It will be interesting to elucidate whether rice OsHMA2 has a role in Zn loading, since its orthologs in *A. thaliana*, AtHMA2, and AtHMA4, were found to be a limiting step in Zn pumping into the developing seed (Olsen et al. 2016).

Another important question that should be explored in the near future is the effect of increased CO_2 in Zn grain levels, along with Fe and protein, which are commonly correlated. CO_2 levels are increasing and should reach 550 ppm in the next few decades (Carter et al. 2007). A meta-analysis of several crops, including rice, estimated that concentrations of Zn, Fe, and protein will decrease when atmospheric levels of CO_2 reach the predicted levels (Myers et al. 2014; Al-Hadeethi et al. 2019) making biofortification even more urgent. It will be necessary to understand why micronutrient density lowers at high CO_2, and which molecular mechanisms are involved, in order to prevent decreases in current grain quality, which are already below our need. The first possible candidate genes involved in CO_2-mediated decrease in Fe accumulation are being identified (Yang and Zhang 2016; Yang et al. 2020a), and it will be key to find out whether similar machinery is involved in reducing levels of both nutrients under high CO_2.

Finally, Zn agronomic biofortification might benefit from the new nanofertilizer technologies that are emerging. Phytonanotechnology, as it has been called, can allow slow, on-demand release of nutrients to plant absorption, reducing adverse effects in plants and the environment. Nanofertilizers would ideally improve plant nutrition and could also help deliver nutrients to specific tissues (for a comprehensive review, see Wang et al. 2016). Indeed, initial work using ZnO particles suggests that nanoparticles can increase Zn levels in rice grains without a yield penalty (Kheyri et al. 2019). However, much more work is necessary, from understanding nanoparticle dynamics in the soil to uptake and delivery in developing seeds.

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Agronomic Approaches for Biofortification of Staple Food Crops

19

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Abstract

The micronutrient malnutrition of iron (Fe), zinc (Zn), and vitamin-A is most prevalent in developing countries, especially in Sub-Saharan Africa and South Asia. Several strategies can prevent and alleviate micronutrient deficiency; the most affordable and accessible are agronomic and genetic biofortification of staple food crops. The plant breeding approach is the most sustainable solution to solve the problem. However, developing new micronutrient dense varieties is a time-consuming process and often micronutrient-deficient soil can limit its effectiveness. Hence, agronomic biofortification by applying Zn- and Fe-containing fertilizers is a short-term solution and represents a useful complementary approach to breeding approaches. Though fertilizer management is crucial to agronomic biofortification, several other agronomic techniques such as seed treatment/coating, microbial inoculation, and cropping system-related approaches are also used as supplementary methodologies. Among fertilizer management, the foliar application required lesser fertilizers and more effective than soil application in terms of grain Fe and Zn enrichment in staple crops. Microbial interventions involving the application of plant growth-promoting rhizobacteria (PGPR) or arbuscular mycorrhiza fungi (AMF) are also known to improve micronutrient acquisition and enhance the micronutrient uptake in grains. The cropping system-related approaches like intercropping (e.g., maize + peanut, wheat + chickpea) and green manuring have been proved the most effective strategy for increasing mobility and uptake of micronutrients (Fe and Zn). However, there are several challenges in adopting agronomic biofortification,

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viz. extra costs of micronutrient fertilizers, issues related to micronutrient bioavailability in the agronomically biofortified food grain and its impact on human nutrition, regulations, and criteria for determining the optimum dose to increase the micronutrient concentration to a desirable level, and concomitant environmental toxicity problems. These issues need to be addressed scientifically through multidisciplinary research approaches.

Keywords

Agronomic biofortification · Bioavailability · Cropping system approaches · Foliar application · Microbial approaches · Seed treatment

1 Introduction

In humans, malnutrition can appear in three forms: hunger and undernourishment, obesity or over nourishment, and micronutrient deficiencies (Ritchie 2017). The micronutrients in human nutrition include vitamins [A, D, E, K, C, B1(thiamine), B2 (riboflavin), B3 (pantothenic acid), niacin, B6 (pyridoxine), folate, biotin, B12 (cobalamin)], fatty acids (linoleic and linolenic), and 17 minerals (Fe, Zn, Cu, Mn, I, F, B, Se, Mo, Ni, Cr, V, Si, As, Li, Sn, Co (in B12) (Gibson 2005). The micronutrients, unlike macronutrients (energy, protein, and fat) are required in small quantities by the human body; however, these are essential for maintaining the normal cellular and molecular functions (West et al. 2012) and for physical and mental development (Ritchie 2017). Globally, micronutrient deficiencies (micronutrient malnutrition) have become an important health issue, with iron, zinc, iodine, and vitamin-A deficiency being most prevalent (Allen et al. 2006; Miller and Welch 2013; Prasad et al. 2016). Micronutrient malnutrition is sometimes termed as "hidden hunger" because micronutrient deficiencies in human health are not always acutely visible (Ritchie 2017). Worldwide over two billion people are affected by micronutrient deficiency (de valença et al. 2017). Among them, pregnant women and children under the age of 5 are the most vulnerable. It had been reported that approximately 500,000 children below 5 years of age die annually due to Zn and Fe deficiencies (Black et al. 2008). As per the WHO estimates, about 250–500 million children suffered from vitamin-A deficiency led blindness of which half these children die within a year due to vision loss (Bailey et al. 2015). As depicted by the Global Hidden Hunger Index (GHHI) map, the hidden hunger is a severe problem mainly in low-income developing countries of Africa and Asia (Fig. 19.1) (Muthayya et al. 2013).

Iron and vitamin-A deficiency are mainly prevalent in developing countries. In Africa, 67.6% of pre-school children and 57.1% of pregnant women and in Southeast Asia 65.5% of pre-school children and 48.2% of pregnant women are suffering from anemia due to iron deficiency (de Benoist et al. 2008). In South and Southeast Asia, about 169 million pre-school children (33% of all pre-school children) and in Sub-Saharan Africa about 104 million (32% of all pre-school children) are suffering

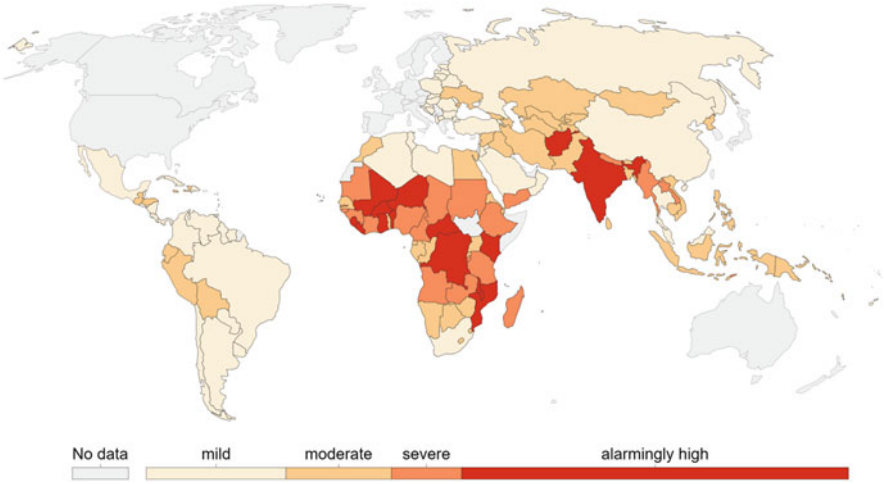

Fig. 19.1 Global hidden hunger index in pre-school children over the period of 1999 to 2009 (Reproduced from Muthayya et al. 2013)

from vitamin-A deficiency (IRRI 2006). In India, about 79.1% of children between the ages of 3 and 6 and 56.2% of married women between the ages of 15 and 49 suffer from anemia (Krishnaswamy 2009). In these low-income developing countries of Asia and Africa, 55% of the dietary energy met through cereals (FAO 2008). These cereal grains are mainly grown in micronutrient-deficient soil, which results in low micronutrient concentration in grains (Cakmak 2008a). The micronutrient deficiencies in the plant, soil, animal, and human are interlinked. There is a close relationship between soil deficiency and human deficiency of Zn and Fe (Shivay and Prasad 2017; Prasad and Shivay 2020). Therefore, micronutrient deficiency in the human diet is more common in developing countries, where micronutrient-deficient soil is prevalent.

Food insecurity, micronutrient-deficient food, and an unhygienic environment with a lack of health services are the major causes that directly contribute to micronutrient malnutrition (Bailey et al. 2015). There are many ways to prevent and alleviate micronutrient deficiency. Miller and Welch (2013) suggested three strategies like mineral supplementation, food fortification, and biofortification for the regions where adequate and diversified dietary intake alone has not met micronutrient requirements. Singh et al. (2016) reported that increasing dietary diversification, mineral supplementation, and food fortification are not effective strategies. Also, people living in developing countries can't access these due to their expensiveness (Bouis 2003; Pfeiffer and McClafferty 2007; Stein et al. 2007; Prasad and Shivay 2020). Therefore, it is suggested to biofortify the crops by applying micronutrient fertilizers, combined with breeding approaches to increase the ability of plant to acquire mineral elements (White and Broadley 2009).

2 Biofortification of Staple Food Crops

Human mainly depends on cereals as a staple food for energy and micronutrients. Staple food crops vary with regions, countries, and communities. The most common staple food crops across the world are rice, wheat, maize, millets, potato, beans, cassava, and sweet potatoes. Nearly 51% of the world's caloric intake is from three food crops such as rice, wheat, and maize (Pariona 2019). Therefore, the enrichment of micronutrients in staple food crops is a major concern (Saltzman et al. 2013). The process of increasing the density of micronutrients in edible parts of crop through genetic and agronomic approaches is known as biofortification (Bouis et al. 2011).

CGIAR Consortium (www.harvestplus.org) had initiated the project The Harvest-Plus with the aim of alleviating deficiencies of mineral nutrients by biofortifying staple food crops with essential minerals and vitamins through plant breeding, an approach considered to be the most economical solution to human micronutrient deficiency (Welch and Graham 2004; Bouis 2007; Cakmak 2008a; Peleg et al. 2009). World Health Organization (WHO) in biofortification program is mainly focusing on three micronutrients such as iron, zinc, and vitamin-A due to its worldwide deficiency. Plant breeding approach is considered the most sustainable solution, but developing new micronutrient-rich plant genotypes is a lengthy and time-consuming process and often the micronutrient-deficient soil can limit its effectiveness (Cakmak 2008a). Moreover, some workers (Garvin et al. 2006; Fan et al. 2008; McDonald et al. 2008; Prasad et al. 2014) found that biofortification through genetic approaches affected grain yields, and Zn concentration and grain yield have negative relation.

Conversely, agronomic biofortification through Zn fertilization resulted in increased grain production and higher Zn concentration in grains at the same time (Prasad et al. 2014; Prasad and Shivay 2020). Moreover, cereal crops are inherently very low in grain Zn and Fe concentrations, and growing them on potentially Zn- and Fe-deficient soils further reduces their concentrations in grain (Cakmak et al. 2010a). Agronomic biofortification, such as applying Zn- and Fe-containing fertilizers, is an effective short-term solution and represents a complementary approach to breeding tools and approaches. Thus, to overcome the Fe and Zn deficiency in food chain the agronomic biofortification approaches need to be taken on priority at the global level (Shivay et al. 2016a).

3 Agronomic Approaches for Biofortification

Agronomic approaches for biofortification involve different strategies to increase the density of micronutrient concentrations in edible parts of crop plants, mainly relying on micronutrient fertilizers and improving the solubility and mobility of micronutrients in the soil (White and Broadley 2009; Prasad and Shivay 2020). Conventionally, the chemical micronutrient fertilizers have been applied to the soil by farmers to improve crop health, and the same strategy can be used to increase the nutrient density in cereal grains (Rengel et al. 1999). In agronomic biofortification,

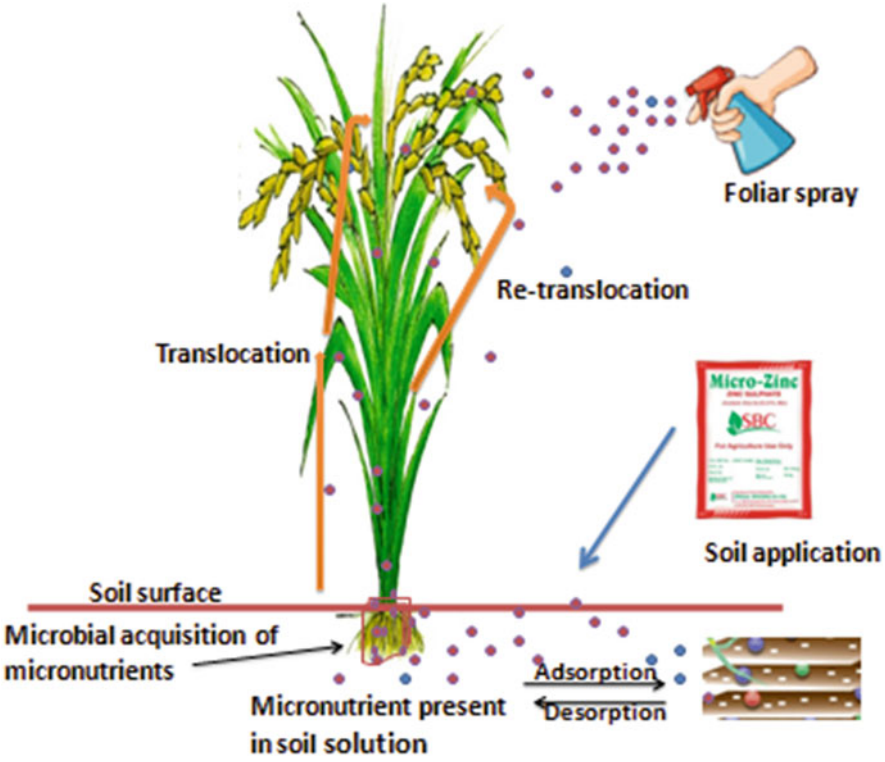

Fig. 19.2 Agronomic biofortification through micronutrient fertilization and microbial acquisition and mobilization of micronutrients; fertilizer (brown circles) applied to the soil and/or plant leaves (through foliar spray), to increase micronutrient contents of the edible part of food crops

the micronutrients are either applied to soil or directly to the leaves. In this process, the plant roots absorb the micronutrients continuously from the soil solution, translocate it into the sink parts, and the micronutrients deposited in leaves and stems (through soil or foliar application) re-translocate into the sink during reproductive stages (Fig. 19.2). However, there are many soil physico-chemical factors that influence the solubility and bioavailability of micronutrients. In general, Zn and Fe bioavailability in alkaline calcareous soil are very low due to high soil pH. The Zn concentration in the soil solution decreases 30- to 45-fold for each unit increase in soil pH within the pH range of 5.5–7.0 (Marschner 1993) thus, increasing the risk of Zn deficiency in plants.

Similarly, the solubility of Fe decreases in alkaline pH ranges. Like high soil pH, soil moisture, and organic matter contents in the soil can determine Zn′s solubility (Graham et al. 1992; Marschner 1993; Alloway 2009). In addition to these, there are many mechanisms responsible for the low solubility and fixation of Zn. These include occlusion in minerals through precipitation of other phases (McLaughlin 2001; Tye et al. 2003), diffusion into micropores and interparticle spaces

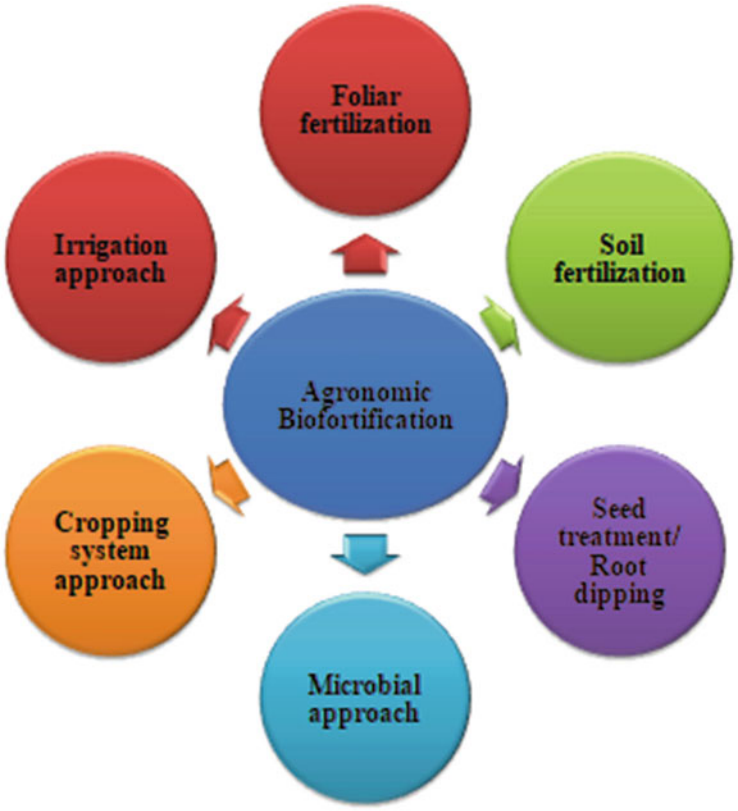

Fig. 19.3 Different agronomic approaches for biofortification of staple food crops

(McLaughlin 2001), solid-phase diffusion (Sparks 1998; Tye et al. 2003), and precipitates including co-precipitation with other metals (Sparks 1998; Almas and Singh 2001). However, agronomic management practices can improve the solubility and mobility of these mineral elements in the soil.

The deployment of optimal fertilizer management practice is central to agronomic biofortification. Apart from that, several other agronomic measures are there that increase the solubility of micronutrients in the soil solution to enhance the micronutrient uptake by root, its translocation, and storage in edible parts of the crop (Fig. 19.3). These agronomic measures are discussed in detail below.

3.1 Soil Fertilization

The nutrient concentration in seed/developing grain in the mother plant depends on soil type, nutrient availability, crop species, and cultivar and to some extent on growing season (Ascher et al. 1994). Hence, when micronutrient fertilizers are

applied to the soil, it increases the micronutrient concentration in soil solution, which becomes easily available for root absorption. In case of macronutrient fertilizers, increased addition leads to diminishing yield response of a crop. Conversely, higher Zn and Fe fertilizer additions increase Zn and Fe concentration in cereal grains (Marschner 1995). Thus, to increase the micronutrients concentration (Fe or Zn) in grain, excess amounts of Zn or Fe fertilizers, which is more than what is required for achieving 90% (or even 100%) of the yield, need to be applied (Rengel et al. 1999).

3.1.1 Rice

Shivay and Prasad (2012) recorded that in Zn-deficient soils, the Zn applied as zinc sulfate heptahydrate or ZnSHH significantly increased rice grain yield and grain Zn concentration (Table 19.1), and also increased harvest index by 2%. The sources of Zn affect its efficiency in terms of increasing yields and nutrient concentration in grain. The fertilizers with higher solubility (e.g., Zn-EDTA and $ZnSO_4$) have usually greater mobility of Zn to the roots zones than insoluble ZnO or fritted Zn (Zaman et al. 2018). The most commonly used Zn fertilizer are Zn sulfate, and it is recommended at 5–25 kg Zn ha^{-1} depending on the application methods, crop species, and nature of soil characteristics (Yilmaz et al. 1997; Cakmak 2008b; Abid et al. 2013; Zhao et al. 2014). A higher fertilizer rate is required in case of alkaline or calcareous soil, Zn deficiency-sensitive crops and the broadcasting method (Alloway 2008). In India, Zn sulfate is commonly used Zn fertilizer due to its higher solubility, easy accessibility, and relatively low cost when compared to other sources of Zn fertilizers (Singh 2008). The recommended dose of $ZnSO_4$ for correcting Zn deficiency in rice is 5 kg Zn ha^{-1} (Gupta 1995a; Rattan et al. 1997). Shivay et al. (2008a, b, c) from New Delhi reported that ZnSHH-coated urea was significantly superior to ZnO-coated urea in increasing Zn concentration in unhusked rice. In contrast, Naik and Das (2008) showed that Zn-EDTA applied at 0.5 kg ha^{-1} resulted in higher Zn accumulation in grain (30.3 mg kg^{-1}), which was better than ZnSHH. Further, they showed that split application was better than a single application of ZnSHH but not in Zn-EDTA. However, the Zn-EDTA use is limited due to its expensiveness.

In contradictory to Zn, soil application of inorganic Fe fertilizers in Fe-deficient soils is usually ineffective because it convert into plant-unavailable Fe (III) forms immediately after application to soil (Rengel et al. 1999). Thus, foliar fertilization of Fe is the most effective for the correction of Fe deficiency in most of the crops (Prasad and Shivay 2020). Long-term application of organic amendments can increase the soluble Fe form by changing soil redox potential (Lindsay 1991). Fulvic acid formed during organic matter decomposition and the siderophores produced by microorganisms are the main substances that increase solubility and bioavailability of Fe to plants (Rengel et al. 1999; Shivay and Mandi 2020). However, Fe availability is not the limiting factor for the lowland rice as rice roots tend to acidify the rhizosphere by releasing proton ion (H^+) and further, submerged soil reduce ferric form iron to ferrous (Prasad et al. 2014).

Table 19.1 Effect of method, source, and rate of Zn application on grain yield of *Basmati* rice (averaged over 2 years)

Treatment (1)	Unhusked rice (t ha^{-1})	Polished rice (PR) (t ha^{-1})	Zn concentration in unhusked rice (mg kg^{-1})	Zn concentration in polished rice (mg kg^{-1})	Agronomic efficiency of Zn (kg grain increased kg^{-1} Zn applied)	Zn harvest index (Zn uptake in grain/Zn uptake in grain + straw) × 100)
Check (no Zn)	3.92	2.74	30.4	26.1	–	16.7
Soil application of 25 kg $ZnSO_4 \cdot 7H_2O$ ha^{-1} (5.3 kg Zn ha^{-1})	5.20	3.64	47.5	40.3	241.5	18.7
One foliar application of 0.2% $ZnSO_4 \cdot 7H_2O$ (1.2 kg Zn ha^{-1})	4.99	3.49	52.6	28.8	901.3	17.5
Soil application of 2% ZnO–coated urea (5.2 kg Zn ha^{-1})	5.13	3.59	44.7	37.39	232.2	19.6
Soil application of 2% $ZnSO_4 \cdot 7H_2O$-coated Urea (5.2 kg Zn ha^{-1})	5.27	3.69	49.7	42.1	259.8	19.1
LSD ($P = 0.05$)	0.45	0.31	4.5	NC	33.4	2.2

NC not computed (Source: Prasad et al. 2014; Shivay and Prasad 2012)

3.1.2 Wheat

The wheat is cultivated mostly in Zn-deficient soil which leads to inherently low grain Zn concentration (Alloway 2009). Foliar application of Zn resulted in a significantly higher Zn concentration in wheat than soil application (Yilmaz et al. 1997; Cakmak et al. 2010a; Cakmak et al. 2010b; Zhang et al. 2010). However, Maqsood et al. (2009) showed that Zn concentration varied from varieties to varieties and recorded 51.7–69.9% variation with 6 mg Zn kg^{-1} soil application. Similarly, Kumar et al. (2018) experimented with red and laterite soil with 23 wheat cultivars and showed that soil application increased Zn concentration from 38.9 to 77.2 mg kg^{-1}. Moreover, Hussain et al. (2012) recorded that soil application of Zn increased grain yield by 29%, whole-grain Zn concentration by 95%, and whole-grain estimated Zn bioavailability by 74%. Cakmak et al. (2010b) suggested that Zn′s combined application through the soil and foliar spray are the most effective for increasing Zn density instead of only soil application.

Zinc and phosphorus have a mutually antagonistic relationship both in plants and soil. Zn application under Zn-deficient soil considerably increases grain Zn and reduces grain P concentration (Erdal 1998). The low P concentration in grain further decreases the phytate concentration in grain and reduces the phytate to Zn molar ratios. Phytate is the primary P storage compound in cereal grains and forms insoluble complexes with Fe and Zn, reducing the bioavailability of Zn and Fe in the human intestine (Wise 1995; Lott et al. 2000).

Soil application of Fe to wheat is not as effective as in case of rice. However, Fe has a positive interaction with nitrogen and sulfur that increases Fe uptake by crop plants. Shivay et al. (2016b, c) reported that applying N and S as sulfur-coated urea resulted in significantly increased Fe concentration in wheat grain. They also found that incremental dose of sulfur significantly increased Fe concentration in wheat grain (Table 19.2). Nitrogen and sulfur application to soil had increased the soil acidity, thereby increasing Fe solubility and availability (Prasad and Shivay 2020).

Similarly, Xu et al. (2012) also recorded that the application of 0, 99, 198, and 297 kg N ha^{-1} to winter wheat resulted in grain Zn concentration of 21.5, 25.1, 30.9, and 37.0 mg kg^{-1}, respectively. Zn concentration in wheat grains also increases with

Table 19.2 Effect of nitrogen [as urea or sulfur-coated urea (SCU)] on Fe and Zn content in wheat grain

Treatment	Fe (mg kg^{-1} grain)	Zn (mg kg^{-1} grain)
Check (0N0S)	150	37.3
130 kg N ha^{-1} as urea	156	39.2
130 kg N ha^{-1} + 3.16 kg S ha^{-1} as SCU	161	40.9
130 kg N ha^{-1} + 6.32 kg S ha^{-1} as SCU	166	42.8
130 kg N ha^{-1} + 9.48 kg S ha^{-1} as SCU	171	43.2
130 kg N ha^{-1} + 12.64 kg S ha^{-1} as SCU	176	43.8
130 kg N ha^{-1} + 15.0 kg S ha^{-1} as SCU	181	44.5
LSD ($P = 0.05$)	4.69	1.90

Source: Prasad and Shivay (2020)

high N application rates as the nitrogen improves root uptake and translocation of Zn (Kutman et al. 2010; Kutman et al. 2011a,b; Singh et al. 2018). Thus, nitrogen and sulfur management practices are as useful as agronomic tool for the biofortification of Zn (Kutman et al. 2010).

3.1.3 Corn

Information on Zn and Fe agronomic biofortification in corn through fertilizers is meagre due to fewer research works. Imran and Rehim (2016) reported that combined application of subsurface banding and foliar spraying of Zn increased grain Zn concentration by 46.8% compared to control. Further, they found increased Zn bioavailability in grain by 52% and decreased phytate concentration. However, Shivay and Prasad (2014) found that the maximum Zn concentration (49.2 mg kg^{-1}) with 5 kg Zn to soil + 1 kg Zn as a foliar spray. It was suggested that the tasselling and flowering initiation are critical stages for foliar spraying of Zn. Manzeke et al. (2014) reported that combined applied Zn-enriched fertilizer and cattle manure and forest leaf litter resulted in significant increase in yield and Zn concentration of corn. As organic substances can complex with metals by its ligand and functional groups, which forms soluble complex, the application of organic matter can improve the availability of Zn in soil (Santos et al. 2010). Apart from Zn, corn has responded positively to selenium biofortification through Se-enriched fertilizer application (Alfthan et al. 2015).

3.2 Foliar Fertilization

Nutrients are delivered to plants through leaves by foliar spraying as plants can absorb soluble compounds and gases through leaves (Kannan 1990). The foliar nutrients pass through the cuticle, the stomata, trichomes, and other specialized epidermal cells to enter the cell's cytoplasm within the leaf (Franke 1967). The upward transport of nutrients in the stem may occur through phloem or xylem, but the translocation of nutrients from leaf toward grain or its downward movement in stem occurs only in the phloem (Rengel et al. 1999). Foliar application of Zn fertilizers results in significant increase of Zn concentration in cereal grains than soil application. However, response to foliar applications varies with crop species. Cakmak and Kutman (2018) observed that wheat was the most response to foliar spray followed by rice and maize.

3.2.1 Rice

Shivay et al. (2010a,b) reported that foliar application of 1.2 kg Zn ha^{-1} and soil application of 5.3 kg Zn ha^{-1} gave equal grain yield and Zn harvest index of rice. But, higher Zn concentration in grain and agronomic efficiency (4-times higher) was observed with foliar application (Table 19.1). Ghasal et al. (2018) observed that combined application of 1.25 kg Zn ha^{-1} as Zn-EDTA + 0.5% foliar spray in aromatic rice variety "PB 1509" recorded higher Zn concentration in white rice kernel, hull, and bran. Phattarakul et al. (2012) in their multi-locational trial across

Table 19.3 Grain yield and relative zinc concentration in unhusked, brown, and white (polished) rice (averaged over 9 site years in China, India, Lao PDR, Thailand, and Turkey)

Characteristic	Control (no Zn)	Soil Zn	Foliar Zn	Soil + foliar Zn	Significance
Grain yield (t ha^{-1})	6.7	7.0	6.9	7.0	NS
Zn in unhusked rice (mg kg^{-1})	18.7	19.1	32.3	34.7	$P < 0.01$
Zn in brown rice (mg kg^{-1})	19.1 (102.1)[a]	20.8 (108.9)	24.4 (75.5)	25.5 (73.5)	$P < 0.01$
Zinc in polished rice (mg kg^{-1})	16.1 (18.1)[b] (84.2)[c]	16.2 (84.8) (77.9)	17.7 (54.8) (72.5)	18.4 (53.0) (72.1)	$P < 0.01$

Source: Phattarakul et al. (2012)
[a]Zn in brown rice expressed as percentage of unhusked rice
[b]Zn in polished rice expressed as percentage of brown rice
[c]Zn in polished rice expressed as percentage of unhusked rice

various countries reported that foliar application of Zn increased the Zn concentration in unhusked rice (whole paddy) grain by 69% in comparison to the soil application, and in some places, it was almost twice that of with soil application. When Zn was foliar applied, only 53–54% of unhusked rice's Zn content was found in polished or white rice compared with 84.8% when Zn was soil applied (Shivay and Prasad 2012; Prasad et al. 2014) (Table 19.3). A more generous portion of Zn remained in the husk in the foliar application case while a lesser portion was stored in white rice. Conversely, the soil-applied Zn fertilizers increased the Zn content in white rice to a greater extent. This phenomenon indicates that the penetration of Zn from the husk into the inner layers of rice endosperm was better with soil Zn application. This might be due to root transport of Zn through the xylem, which plays a vital role for Zn accumulation in rice grain than re-translocation of Zn from the leaves (Jiang et al. 2007; Palmgren et al. 2008). Besides, a high dose of Zn was applied to soil compared with that on foliage. Further, the removal of husk, aleurone layer, and germ layer of rice during hulling and milling decreases the Zn concentration up to 16.2%–48.2% in different rice genotypes, which aggravate the problem of Zn malnutrition (Saenchai et al. 2012).

Foliar spray is effective in specific growth stages of crops for achieving higher Zn concentration in grain (Welch et al. 2013). It was observed that foliar application after "milking" stage of crops was more effective for Zn loading into the grain (Cakmak et al. 1994; Shivay and Prasad 2014). Moreover, Boonchuay et al. (2013) recorded that foliar Zn sprays at four stages (panicle initiation, booting, 1 and 2 week after flowering) resulted in the highest Zn concentration in rice than early growth stages.

Fe biofortification by foliar spraying increased a significant amount of Fe in brown rice than soil application methods (Aciksoz et al. 2011). Fe concentration in rice grain could be increased by 20%–43% by foliar application of Fe (Table 19.4). Prasad and Shivay (2018) suggested that Fe foliar spray to all cereal grains is required to alleviate Fe deficiency in a country like India, specifically for people

Table 19.4 Iron concentration in cereal grains as influenced by foliar iron fertilization

Crop	Country-location	Treatment (kg ha^{-1})	Fe in grain (mg kg^{-1})	% increase
Rice (rough)	India-New Delhi	0 Fe	88.5B	–
		6 Fe	105.6A	19.3
Rice (rough)	India-New Delhi	0 Fe	37.6B	43.4
		15 Fe	53.9A	5 varieties
Rice (rough)	India-New Delhi	0 Fe	18.5B	30.3
		1.5 Fe	24.1A	5 varieties
Wheat	India-New Delhi	0 Fe	37.54B	–
		9 Fe	43.30A	15.4
Wheat	Romania-Timisoara	0 Fe	42.94B	–
		0.333 Fe	54.74A	27.5
Wheat	China	0 Fe	29.5B	18.3–28.1
		1.84 Fe	34.9–37.8A	3 varieties

Values followed by different letters (A, B) differed significantly
Source: Prasad and Shivay (2020)

living below the poverty line and who cannot access balanced diet or dietary supplements. Foliar-applied Fe absorbed through leaf epidermis remobilized and translocated into the grain via the phloem, but the Fe loading into the phloem was limited (Borg et al. 2009; Fageria et al. 2009; Kobayashi and Nishizawa 2012). Nicotianamine (NA) is an organic chelator that forms organo-metal complex with Fe and Zn and also acts as a transporter and maintains homeostasis in plants (Takahashi et al. 2003). Therefore, the combined foliar application of nicotianamine (NA) and $FeSO_4$ enhanced the Fe concentration in brown rice (Yuan et al. 2012). Further, Wei et al. (2012) recorded that the combined foliar application of $FeSO_4$ and nicotianamine (NA) resulted in increased Fe concentration by 29.9% in polished rice grain and also increased Fe bioavailability by 20.9%. This might be due to the reduction of phytic acid as it is inversely related to increasing Fe or Zn.

3.2.2 Wheat

Foliar-applied Zn is absorbed by leaf epidermis and readily translocated into developing grains in wheat as it is phloem-mobile in nature (Haslett et al. 2001; Erenoglu et al. 2011). Zou et al. (2012) in their multi-locational trial across the world reported that foliar Zn application alone and in combination with soil application recorded 84% and 90% higher Zn concentration in grain, respectively, as compared to control treatment. (Table 19.5). Dhaliwal et al. (2019) reported that foliar Zn application increased Zn content in bread wheat, triticale, and durum wheat cultivars grains varying from 31.0 to 63.0, 29.3 to 61.8, and 30.2 to 62.4 mg kg^{-1}, respectively. Ghasal et al. (2017) showed that soil + foliar application of Zn was superior over soil application alone, and the soil application of 1.25 kg Zn ha^{-1} (Zn-EDTA) + 0.5% foliar spray recorded the highest Zn concentration in grain and straw. Thus, combining foliar and soil-applied application is the most effective application method for loading Zn in food grains and avoiding native Zn depletion from the soil.

Table 19.5 Increase in Zn concentration (mg kg^{-1}) in wheat grain due to Zn application at field capacity in seven countries (23 site-year trials) (figures in parentheses are % increase over check)

Country	Location (number of years)	Variety	Check (control)	Soil Zn	Foliar Zn	Soil + foliar Zn	Significance
China	Guzhou (2)	Kenong 9204	28.6	36.6 (28.1)	45.7 (60.0)	52.9 (85.1)	**
	Yangzhou (2)	Jonmai 47	19.1	21.5 (12.8)	28.9 (51.6)	28.2 (47.9)	**
India	Varanasi (1)	HUW 234	29.0	32.0 (10.3)	44.0 (51.7)	47.0 (62.1)	**
	Kapurthala (2)	DBW 17	40.2	41.1 (2.2)	57.9 (44.1)	57.2 (42.3)	**
	Ludhiana (2)	DBW 17	26.4	33.5 (26.9)	59.6 (125.9)	58.9 (123.1)	**
Kazakhstan	Shortandy (1)	Akmela 2	21.5	29.5 (37.2)	66.5 (209.3)	76.5 (255.8)	**
Mexico	Yaqui Valley (1)	Kronstad	26.0	25.0 (19.0)	43.0 (104.8)	45.0 (114.3)	**
Pakistan	Ayub (1)	Sehar 2006	27.0	25.3 (−6.2)	48.2 (78.5)	44.6 (65.2)	*
	Faislabad (1)	Auqab 2000	29.0	29.0 (0.0)	60.0 (106.9)	59.0 (103.4)	**
	Muridke I (2)	Sehar 2006	36.6	35.8 (−2.0)	48.3 (32.1)	48.1 (31.4)	*
	Muridke II (2)	Sehar 2006	38.9	46.4 (19.2)	52.2 (34.2)	52.0 (33.6)	*
Turkey	Eskisehir (2)	Bezostaya 1	25.8	26.0 (0.7)	43.4 (68.4)	43.3 (68.0)	**
	Konya (2)	Bezostaya 1	12.8	12.9 (0.7)	25.7 (101.1)	27.3 (113.7)	**
Zambia	Chisamba (1)	Rorrie II	23.0	24.0 (0.4)	–	43.0 (86.9)	**
Average			27.4	30.5 (11.3)	48.0 (75.2)	49.0 (78.8)	**

Significance at $*P = 0.05$, $**P = 0.01$, or less
Soil pH ranged from 7.5 to 8.2, except at Chisamba, where it was 5.7
Soil Zn application: 50 kg $ZnSO_4 \cdot 7H_2O$; foliar: two applications; 0.05% (w/v) aqueous solution at 600–800 L ha^{-1} in the later afternoon; soil + foliar: combination of soil and foliar application
Source: Zou et al. (2012)

The most commonly applied source of foliar Zn applications to wheat is Zn sulfate ($ZnSO_4$) and EDTA-chelated Zn. Zn sulfate is equally effective as Zn-EDTA for correcting Zn deficiency and increasing Zn concentrations in crop plants (Cakmak and Kutman 2018). Moreover, the $ZnSO_4$ application is most economical as compared to Zn-EDTA and later is very expensive. Foliar Zn application at the reproductive stage rather than the vegetative stage, preferably during grain-filling are very effective for loading of Zn in grains (Cakmak et al. 2010a; Boonchuay et al. 2013; Abdoli et al. 2014).

Zn is mainly localized and concentrated in the aleurone and embryo parts of wheat grain, and the endosperm contains a lesser amount of Zn (Ozturk et al. 2006; Cakmak and Kutman 2018). However, the aleurone and embryo layers are rich in phytate, while the endosperm contains very little concentration (Pomeranz 1988; Lehrfeld and Wu 1991; Prom-u-Thai et al. 2008). Phytate is a compound that reduces the bioavailability and limits the intestinal absorption of Zn. So, it implies that although the endosperm has very little concentration of Zn, it is potentially bioavailable and endosperm constitutes the central part of white flour, which is commonly consumed by the people. The agronomic biofortification of wheat through Zn fertilization increases the density of Zn in the whole-grain and also in the endosperm (Cakmak et al. 2010a; Kutman et al. 2011a). Grain phytate content can be reduced by increasing Zn concentration in grain that lowers the phytate: Zn ratio, thereby increases the bioavailability (Cakmak 2008a). This can be achieved by Zn fertilization.

The Fe concentration in wheat can also be increased by 15–28% by foliar application of Fe (Table 19.4). Recently, Zou et al. (2019) assessed the effect of a combined spray of Zn, Se, Fe, and I on grain micronutrient concentrations of different wheat cultivars in six countries (China, India, Mexico, Pakistan, South Africa, and Turkey) over 2 years. They recorded that Zn foliar spray alone resulted in increased Zn concentration from 28.6 to 46.0 mg kg^{-1}, whereas the combined spray of Zn, Se, Fe, and I increased Zn concentration up to 47.1 mg kg^{-1}. Moreover, they found that the combined spray of Zn, Se, Fe, and I increased Fe concentration in grain by 12%, and similar results were found for Se and I concentration. Thus, the combined application of micronutrients can be an effective strategy to biofortify wheat simultaneously with Zn, I, Se, and Fe.

3.2.3 Chickpea

The grain legumes are an important source of protein-rich diet in developing countries, where a majority of the population is vegetarian, however, pulses have not received much attention for agronomic biofortification (Prasad 2009). Chickpea (*Cicer arietinum* L.), also known as Bengal gram or garbanzo, is the third important pulse crop after dry beans and peas. In many developing countries, especially in South Asian countries, chickpea is one of the major pulse crops. Pal et al. (2019) from India studied the effects of soil and foliar application of Zn and foliar application of urea on Zn and Fe accumulation in chickpea grains (Table 19.6). They reported that combined application of $ZnSO_4$ at 25 kg ha^{-1} soil-applied plus 0.5% foliar spray of $ZnSO_4$ at flowering and pod formation stages resulted in 44.7 mg kg^{-1}

Table 19.6 Effect of zinc and urea application on zinc and iron content in grains of chickpea

Treatment	Zn concentration in grain (mg kg^{-1})	Zn concentration in straw (mg kg^{-1})	Iron concentration in grain (mg kg^{-1})	Reference
Zinc application				Shivay et al. (2015)
Control	36.3	13.5	–	
NPK	41.4	17.1	–	
NPK + ZnSHH soil at 5 kg Zn ha^{-1}	50.7	21.3	–	
NPK + ZnSHH, three sprays	57.1	31.2	–	
NPK + Zn-EDTA at 2.5 kg Zn ha^{-1}	51.3	23.4	–	
NPK + Zn-EDTA, three sprays	63.5	32.6	–	
LSD ($P = 0.05$)	3.33	1.81	–	
Control	28.99	–	54.64	Pal et al. (2019)
Soil application of $ZnSO_4$ at 25 kg ha^{-1} at sowing	35.29	–	58.11	
Foliar spray of $ZnSO_4$ at 0.5% at flowering and pod formation stages	41.44	–	60.19	
Soil application of $ZnSO_4$ at 25 kg ha^{-1} at sowing + foliar spray of $ZnSO_4$ at 0.5% at flowering and pod formation stages	44.69	–	62.88	
LSD ($P = 0.05$)	1.96	–	3.43	
Urea application				
Control	35.11	–	56.33	
Foliar spray of urea at 2% at flowering stage	38.51	–	59.97	
Foliar spray of urea at 2% at flowering and pod formation stages	40.26	–	61.95	
LSD ($P = 0.05$)	1.38	–	2.43	

and 62.9 mg kg^{-1} Zn and Fe concentration in grain, respectively. Further, they recorded that 2% urea application at flowering and pod formation stages resulted in higher Zn and Fe content. Fe, Zn, and N have positive interaction, and they share similar mechanisms for translocation from source to sink (Pal et al. 2019). Therefore, combined application of Zn fertilizer and urea improved Zn content in chickpea grain than the sole application separately.

In another study, Shivay et al. (2015) found that the Zn application either soil-applied or foliar in the form of ZnSHH or Zn-EDTA increased Zn concentration in grain and straw of chickpea (Table 19.6). Three sprays of ZnSHH resulted in significantly higher Zn in grain than soil application. Among the two sources of

Zn, foliar spray of Zn-EDTA recorded significantly higher Zn concentration in grain than ZnSHH.

3.3 Seed Treatment/Roots Dipping

Foliar spray and soil applications have been effective in increasing yield and micronutrient content in food grain, but the extra fertilizer cost sometimes discourages the resource-poor farmers from adopting these practices (Johnson et al. 2005). Seed treatments with micronutrients before sowing of crops are known as seed priming. The seed priming and seed coating with essential micronutrients are easy, cost-effective, and practical approach in alternative to foliar and soil-applied micronutrients (Farooq et al. 2012). Seed priming requires lesser quantity, easy to apply, and shows vigorous seedling development (Singh et al. 2003). In micronutrient seed priming (nutripriming), seeds are soaked in desired micronutrient solutions for a certain period of time for the metabolic activities to start and then re-dried in the shade (Bradford 1986; Imran et al. 2004; Singh 2007; Farooq et al. 2012). Seed priming with 1% $ZnSO_4$ solution for 16 h substantially improved crop growth, grain yield, and grain Zn content in maize (Harris et al. 2007). Further, Harris et al. (2008) recorded that seed priming with Zn, increased grain Zn content by 12% in wheat and by 29% in chickpea. Moreover, Shivay et al. (2013) found that zinc-coated oat seeds (Zn as ZnO or Zn sulfate at 2 kg per 100 kg seeds) resulted in higher Zn concentration (32 mg kg^{-1}) in grain as compared with equal dose of soil application (25 mg kg^{-1}).

Seed priming with Zn is more effective in increasing grain yield and cost-effective when grown on Zn-deficient soils. However, seed priming cannot increase Zn content to that extent as increased by foliar and soil application (Yilmaz et al. 1997, 1998). Therefore, seed priming along with Zn fertilization, either soil-applied or foliar or both are required for increasing grain yield and Zn content in grain (Stomph et al. 2011; Zaman et al. 2018). Seed priming with Zn can also improve crop germination and growth and suppress various soil-borne diseases. Ajouri et al. (2004) showed that seed priming with Zn resulted in better germination and development of vigorous and healthy seedling in barley. During the seed germination process and early seedling development stage, Zn plays a vital role in physiological processes like protein synthesis, cell elongation, membrane function, and resistance to abiotic stresses (Cakmak 2000; Ozturk et al. 2006). Besides, Zn suppresses important fungal diseases of wheat, including *Fusarium* crown rot, *Rhizoctonia cerealis* "winter-kill," and "take-all" caused by *Gaeumannomyces graminis* (Brennan, 1992; Grewal et al. 1996; Braun, 1999).

In general, rice seedlings are soaked with micronutrients solution for specific times before transplanting to overcome Zn deficiency. Seedlings soaked with ZnO suspension had been proved useful for correcting Zn deficiency in rice. Das et al. (2019) found that seedling roots soaked with 2% ZnO slurry resulted in higher plant height, tiller numbers, and greenness index. This might be due to better Zn nutrition as roots directly contacted Zn slurry. However, in later stages of crop

growth, foliar spray of Zn alone or combined with soil application is most effective than root dipping in increasing crop growth, yield, and enrichment of Zn in rice (Rashid 2001; Robson 2012). Thus, it is suggested that roots dipping method of Zn application should be combined with other application methods to get better crop growth, yield, and higher Zn content.

3.4 Microbial Approach

Microbial interventions are gaining importance as a strategy for enhancing the solubility and availability of micronutrients in the rhizosphere and the plant's nutrient uptake. Though there are large amount of Fe and Zn present in the earth's crust, these are unavailable to plants because of numerous edaphic factors like high $CaCO_3$, neutral or alkaline pH, low redox conditions, etc. To survive on this micronutrient-deficient soil, plants have developed their own mechanism like phytosiderophore release or chelators secretions or organic acid production to improve micronutrient availability in the rhizosphere. But these intrinsic strategies are not effective in all cases for increasing micronutrient availability to plant in micronutrient-deficient soils (Singh and Prasanna 2020). Hence, microbial approaches play a crucial for the biofortification of Zn and Fe in cereal grains (Gosal et al. 2010; Rana et al. 2012a; Sharma et al. 2012).

Singh and Prasanna (2020) have elucidated different mechanisms in their review through which microbes can increase the bioavailability of Zn and Fe in the soil and increase the density of Fe and Zn in food grains due to enhanced absorption and translocation. These include the following: (1) Siderophores and other chelating substances production; (2) organic acid secretion and proton extrusion; (3) modification in root morphology and anatomy; (4) upregulation of Zn and Fe transporters; (5) reduction of phytic acids or anti-nutritional factors in food grains; (6) secretion of phenolics and related reducing moieties; and (7) secretion of phytohormones like signaling molecules. Most of the research findings related to microbial interventions involve either plant growth-promoting microorganisms (PGPMs) or arbuscular mycorrhizal fungi (AMF).

Plant growth-promoting rhizobacteria (PGPR) include a wide variety of soil bacteria. PGPR promotes root function, enhances growth and development, and suppresses disease by associating with host plants. Nevertheless, PGPR's major beneficial role is to increase the mobility, uptake, and enrichment of micronutrients in the plant (Cakmakci et al. 2006; Glick 1995). Rana et al. (2012b) recorded that the combined application of *Bacillus sp*. and *Providencia sp.* was resulted in a significant increase in Zn and Fe concentration of wheat grains. Also, Rana et al. (2015) reported that a combined inoculation of *Providencia* sp. + *Brevundimonas diminuta* + *Ochrobactrum anthropi* resulted a significant uptake of Fe, Zn, Cu, and Mn in rice grains (Table 19.7). They also recorded that inoculum *Providencia* sp. resulted in the highest wheat grain yield (5.23 Mg ha^{-1}) and significantly higher Fe and Cu concentration (44–45%) in the grains. Tariq et al. (2007) showed that Zn solubility could be increased by applied PGPR consortium (containing

Table 19.7 Effect of microbial inoculants treatment on micronutrient concentration in rice and maize crop

Crop	Treatment	Zinc concentration in leaves (μg g^{-1})	Zn concentration in grain (mg kg^{-1})	Fe concentration in grain (mg kg^{-1})	Reference
Rice	Absolute control	–	21.35	132.93	Rana et al. (2015)
	$N_{120}P_{60}K_{60}$	–	31.65	152.23	
	$N_{90}P_{60}K_{60}$ + *Brevundimonas diminuta* + *Ochrobactrum anthropi* + *Bacillus pumilus*	–	36.62	176.25	
	LSD ($P < 0.05$)	–	1.38	5.82	
Maize	Control (uninoculated)	77.48	–	–	Prasanna et al. (2015)
	Anabaena–Trichoderma biofilmed Formulation	87.90	–	–	
	Anabaena–Nostoc spp. Consortial formulation	94.97	–	–	
	Anabaena–Azotobacter biofilmed Formulation	107.01	–	–	
	LSD ($P < 0.05$)	3.38	–	–	

Pseudomonas sp. and other strains of PGPR) and that may increase Zn concentration up to 157%. Further, Prasanna et al. (2015) found that microbial inoculation of *Anabaena–Azotobacter* biofilm resulted in significantly higher Zn concentration in flag leaf (Table 19.7). Published literature illustrates the promise of diverse groups of microorganisms, including endophytes, in enhancing the Zn and Fe availability in soil and translocation to grains (Adak et al. 2016; Singh et al. 2019; Singh and Prasanna 2020).

About 80% of terrestrial plant species form a symbiotic relationship with arbuscular mycorrhizal fungi (AMF); the mycorrhizal fungi colonize the plant's roots and exchange soil-derived nutrients for plant-derived photosynthates and lipids (Smith and Read 2008; Kaiser et al. 2015). AMF can improve the uptake of relatively immobile nutrients in the soil (e.g., P, Zn, Fe, Cu, and K) (Pellegrino and Bedini 2014; Pellegrino et al. 2015; Watts-Williams and Cavagnaro 2018). Therefore, it enhances plant growth and productivity. The colonization of arbuscular mycorrhizal fungi (AMF) in upland plants can enhance nutrient uptake by developing the extensive surface area or network in the soil-plant system through external hyphae (Smith and Read 1997). Thus, AMF is the most crucial mycorrhiza in the agriculture production system and closely relates to human nutrition (Singh et al. 2016). In Zn-deficient soil, the beneficial effect of AMF in improving the acquisition and uptake of Zn was found in pigeon pea (Wellings et al. 1991), low land rice (Purakayastha and Chhonkar 2001), wheat (Ryan and Angus 2003), and tomato (Cavagnaro et al. 2010). The Zn nutrition improvement is due to the direct uptake of Zn by AMF and/or indirect effects through morphological and physiological alteration of plant roots through colonization by AMF (Cavagnaro 2008).

Rice plants, both in upland and lowland, can form mycorrhizal associations (Ilag et al. 1987; Gupta 1995b). Gao et al. (2007) observed that AM fungi enhanced Zn uptake in aerobic rice under Zn-deficient soil. However, the increase in Zn uptake by AM fungi was only in genotypes with a low inherent Zn uptake. Coccina et al. (2019) reported that Zn uptake by mycorrhiza contributed 24.3% and 12.7% of total above-ground Zn in wheat and barley, respectively. In addition to this, AMF increased the grain yield of bread wheat. Similarly, Mäder et al. (2011) found a substantial increase in Zn and Mn concentrations through natural AMF consortium and combined inoculation of two *Pseudomonas* strains.

3.5 Cropping System Approaches

3.5.1 Intercropping

Intercropping is growing two or more crops with different rooting pattern and growth habits simultaneously in the same field with a definite row ratio. Intercropping is an important cultural practice in agriculture because it utilizes the resources effectively and enhances crop productivity significantly compared to the monoculture crops (Li et al. 1999, 2007). Intercropping has also a crucial role in improving solubility and mobility of micronutrients uptake, thereby increasing the root uptake. This is achieved through the inter-specific root interactions in the

Table 19.8 The effects of intercropping on Fe and Zn concentrations in plant tissue (mg kg^{-1} dry weight) of peanut, wheat, and chickpea

Cropping system	Micronutrients concentrations in plant tissue (mg kg^{-1})			Reference
	Shoots	Roots	Seeds	
Fe				
Peanut monocropped	28.0 b	159.5b	22.2b	Zuo et al. (2000)
Peanut intercropped (with maize)	65.5 a	203.1a	31.8 a	
Wheat monocropped	28.69 b	–	36.58 b	Gunes et al. (2007)
Wheat intercropped	40.31 a	–	46.13 a	
Chickpea monocropped	70.65 b	–	18.75 b	
Chickpea intercropped	80.11 b	–	22.75 a	
Zn				
Peanut monocropping	10.4 b	–	–	Inal et al. (2007)
Maize + Peanut intercropping	26.2 a	–	–	
Wheat monocropped	5.71 b	–	25.09 b	Gunes et al. (2007)
Wheat intercropped	9.45 a	–	27.10 b	
Chickpea monocropped	5.01 b	–	10.67 b	
Chickpea intercropped	13.63 a	–	30.05 a	

Values followed by different letters (a, b) differed significantly

rhizosphere (Wasaki et al. 2003; Li et al. 2004; Zuo and Zhang 2009). Intercropping of dicot with graminaceous monocot species increased Zn and Fe uptake in dicot plant. Therefore, the growing of monocot and dicot enhanced micronutrients enrichment in dicot crop plants. Zuo and Zhang (2009) observed that peanut Fe chlorosis was corrected by intercropping with maize in calcareous soil of Henan province, China. These phenomena point out that the maize rhizospheric effect is linked with the improved Fe nutritional status of peanut under field conditions. Zuo et al. (2000) found that the Fe concentration in roots, shoots, and seeds of peanut plants grown in the intercropping system without root barriers were 1.3, 2.3, and 1.4 times higher, respectively, than those of peanut plants grown with root barriers. The maize crop increased the Fe bioavailability and enhanced Zn content in the peanut (Inal et al. 2007) (Table 19.8)**.** Similarly, Gunes et al. (2007) recorded that intercropping between wheat and chickpea resulted in increased Fe content in wheat grain and Fe and Zn content in chickpea grain in field experiments. Zuo and Zhang (2009) suggested the following intercroppings for the enrichment of micronutrients; maize (*Zea mays* L.) + peanut (*Arachis hypogaea* L.), guava (*Psidium guajava*) + sorghum (*Sorghum bicolor*), or maize and chickpea (*Cicer arietinum*) + wheat (*Triticum aestivum*).

Dicot plants species such as peanut and chickpea followed "Strategy I" mechanisms in response to Fe deficiency in which the released protons from the roots cause acidification of the rhizosphere and therefore increase the ferric reductase activity of roots (Römheld and Marschner 1986; Zuo et al. 2000; Zuo et al. 2003). In contrast, the graminaceous plant species followed "Strategy II" mechanism in response to Fe and Zn deficiency, where phytosiderophores are released to improve

the bioavailability of Zn and Fe to the plant roots (Marschner 1998). The phytosiderophores increase the solubility of Fe and Zn by chelation (Rengel 2002; Schmidt 2003; Inal et al. 2007). The graminaceous species can produce higher levels of phytosiderophores even in Fe-deficient calcareous soil, which enhanced iron uptake (Suzuki et al. 2006). This also increased the Fe and Zn uptake in peanut crops/dicot plants when intercropped with monocot species.

3.5.2 Crop Rotation

Crop rotation is the growing of different crop varieties or species sequentially on the same land. Crop rotation practice (especially with the leguminous crop) improves soil chemical and physical fertility, increases water and nutrient use efficiency, and reduces weed infestations and diseases-pest. But, the effect of crop rotation, particularly on soil micronutrients content and its availability, is yet to be studied in detail. Karlen et al. (1994) showed that crop rotation and cover crops might increase the availability of Fe, Cu, and Zn because of microbiologically enhanced chelation. Jat (2010) showed that significantly higher Zn concentration in grain was recorded when aromatic hybrid rice was grown after incorporating cowpea and mungbean residues, which were significantly better than summer fallow.

3.5.3 Green Manuring

Green manuring refers to the soil incorporation of any green manure crops while they are green or soon after they flower. Green manuring has significant effects on soil physical, chemical, and biological properties. But, notably, soil pH is reduced because of the decomposition of organic matter, which produces organic acid and generates CO_2 (Singh et al. 1992; Buragohain et al. 2017). Yadav and Singh (1986), from their 12-year long-term experiments, reported that regular soil incorporation of green manure crops reduced soil pH with time. The low soil pH enhances the availability of most of the micronutrients in the soil; green manure incorporation into the soil increases the availability of diethylenetriamine-pentaacetate (DTPA)-extractable Fe and Zn (Nayyar and Chhibba 2000). The green manure crop draws up the nutrients from deep soil layers and is held inside the plant and recycled back to the soil upon decomposition. Further, the legume green manure between successive crop growth increases the soil organic matter (Pung et al. 2004), which stimulates microbial activities and mineralization of micronutrients (Eriksen 2005).

The rice-wheat is a major cropping system in the Indo-Gangetic plains of India. A window period of 70–80 days before sowing/transplanting rice crop in the rice-wheat system provides an opportunity for growing short-duration cowpea, mungbean (*Vigna radiata* L.), or other green manure crops (Jat et al. 2011). The regular incorporation of dual-purpose summer legumes (cowpea and mungbean) or other green manure crops before transplanting rice may improve not only the soil physico-chemical properties, but also enhanced availability of macro- and micronutrients in the soil. Many studies (Jat et al. 2011, 2013, 2014; Pooniya and Shivay 2011, 2013, 2015 and Singh and Shivay 2013, 2015) showed that summer green-manuring crops residue incorporation in *Basmati* rice-wheat cropping sequence had positive effects on Zn uptake, Zn concentration of grain and straw in

Table 19.9 Effect of summer green-manuring crops residue incorporation on zinc concentration of grain and straw in rice and wheat crop

Crop	Treatment (kg ha^{-1})	Zn in grain (mg kg^{-1})	Zn in straw (mg kg^{-1})	% increase in grain	% increase in straw	Reference
Basmati rice	Summer fallow	23.7 b	151.0 b	–	–	Singh and Shivay (2015)
	Sesbania aculeata	32.4 a	172.0 a	36.7	13.9	
Durum wheat	Summer fallow	35.6 b	111.6 b	–	–	Singh and Shivay (2013)
	Sesbania aculeata	42.8 a	125.7 a	20.2	12.6	
Basmati rice	Summer fallow	18.9 b	146.9 b	–	–	Pooniya and Shivay (2013)
	Sesbania aculeata	21.8 a	164.7 a	15.3	12.1	
Basmati rice	Summer fallow	17.6 b	145.9 b	–	–	Jat et al. (2011)
	Cowpea	19.6 a	156.9 a	11.4	7.5	
Wheat	Summer fallow	27.6 b	107.9 b	–	–	Jat et al. (2013)
	Cowpea	33.9 a	114.7 a	22.8	6.3	

Values followed by different letters (a, b) differed significantly

both the crops (Table 19.9). It increases Zn recovery efficiency as well as the Zn harvest index of both the crops.

Singh and Shivay (2015) found that *Sesbania aculeata* incorporation and application of EDTA-chelated Zn (12% Zn) in the rice-wheat cropping system significantly increased the Zn concentration and uptake in grain and straw of rice. Singh and Shivay (2013) found that the residual effect of summer green manures (*Sesbania aculeata*) significantly increased the Zn concentration in grain and straw and improved quality parameters of durum wheat. The leguminous green manure crop residue incorporation to the soil increases the N supply in the soil, thereby N availability, which in turn positively stimulated the Zn uptake by the wheat plant and Zn accumulation in grains (Kutman et al. 2010). Similarly, Pooniya and Shivay (2013) reported significantly higher Zn concentration in grain and straw of rice using *Sesbania aculeata* green manuring and application of 0.2% foliar spray of $ZnSO_4 \cdot H_2O$. Jat et al. (2013) reported the significant residual effect of cowpea residue incorporation on grain and straw Zn concentrations and Zn uptake of succeeding wheat crop under rice-wheat cropping system. Further, they found that the significantly higher Zn content and uptake in grain when aromatic hybrid rice was grown after incorporating cowpea residue.

3.6 Irrigation

Irrigation practices influence micronutrient solubility and availability in the soil; hence, it affects the plant's nutrient uptake. Micronutrients can be directly applied to the soil and crop through irrigation. Moreover, the proper water management practices can alter micronutrient availability in soil solution depending upon the soil types. One such example of biofortification through irrigation is the iodization of irrigation water in southern Xinjiang Province of China to solve the severe iodine deficiency problem. Standard interventions (like iodized salt and iodine-in-oil capsules) were not able to solve this problem (Ren et al. 2008). Therefore, potassium iodate was dripped into irrigation water canals, which was then distributed into soil, crops, animals, and people. Further, three-fold increase in soil iodine and two-fold increase in iodine content of wheat crop were recorded after dripping 5% potassium iodate into irrigation canals. There was also an increase in iodine levels in animals and humans relying on local crops for food (Cao et al. 1994). Hence, micronutrients enrichment through irrigation water proved to be a practically feasible and cost-effective method.

4 Challenges of Agronomic Biofortification

- The extra-cost of micronutrient fertilizer.
  The fertilizer cost, along with the application cost, may incur additional expenditure to resource-poor farmers in developing countries. Again the agronomic biofortification might not have a clear economic return unless the crop productivity is limited by Zn deficiency or there is a premium price for biofortified grain (Cakmak and Kutman 2018). However, adequate research findings support the fact that the costs of Zn fertilizer application are small compared with the economic returns through increases in yield and the public health benefits (Harris et al. 2007; Shivay et al. 2008a; Manzeke et al. 2014; Joy et al. 2016). Moreover, the total cost of fertilizer can be skipped by applying micronutrient fertilizer together with pesticides that need to be applied anyway (Ortiz-Monasterio et al. 2015; Wang et al. 2015; Ram et al. 2016).
- Determination of optimum dose and the micronutrient toxicity.
  After setting appropriate target levels for the micronutrient content in biofortified staple food, optimum fertilizer dose needs to be determined to achieve the target. But, great difficulty exists in setting appropriate target levels as it differs with population, processing practices and further, the bioavailability of micronutrients in food grain varies with different application methods.

  If the micronutrient fertilizers are applied continuously in large amounts, these elements accumulate over time and cause toxicity. However, the application of micronutrient (like Zn, Fe) fertilizers have a minimal negative impact on the environment (Broadley et al. 2007; Alloway 2009) because most of the micronutrients are strongly bound in the soil and are not susceptible to leaching (de Valença et al. 2017). Moreover, the foliar Zn fertilizer solution contains

around 1 kg Zn ha^{-1}, which is considered as completely safe for the ecosystem (Cakmak et al. 2010a; Boonchuay et al. 2013; Ram et al. 2016). Focused research needs to be done to determine optimum fertilizer application rates to increase grain micronutrient concentration to a desirable level while minimizing environmental pollution and toxicity.

- Issue of bioavailability from agronomic biofortified crop.
  Micronutrients (Fe or Zn) are mostly localized and concentrated in the germ and/or aleurone layers of the grain, while a lesser amount is present in the endosperm. But, during milling operation and other processing steps, these fractions are lost. Thus, micronutrient content is reduced substantially and becomes unavailable to the large population in developing countries who consume refined grains (Hotz and McClafferty 2007).

  Apart from these external factors, internal factors that hinder the bioavailability of micronutrients in humans are concerns. The aleurone layer and embryo of both the rice and wheat are rich in phytate, while the endosperm contains very less concentration of it (Pomeranz 1988; Lehrfeld and Wu 1991; Prom-u-Thai et al. 2008), and the phytate compound reduces the bioavailability of Zn and Fe and limits its intestinal absorption. Diets with phytate/Zn molar ratios above 15 are associated with reduced Zn bioavailability and cause Zn deficiency in humans (Bindra et al. 1986; Gibson 2005; Prasad et al. 2013). Hence, extensive research needs to be done to increase the micronutrient content in endosperm and to reduce the phytate content in the overall grain so that the bioavailability of Zn and Fe can increase.
- Impact of agronomic biofortification on human nutrition and health status.
  There is a dearth of research findings quantifying the direct impact of agronomically biofortified food crops consumption on human health. Agronomic biofortification can increase micronutrient contents in crops, but literature regarding the bioavailability of micronutrient and its effect on dietary intake and human health are scarce (Joy et al. 2014). However, in Finland, nationwide agronomic Se biofortification through the addition of Se to NPK fertilizers resulted in a spectacular increase in cereal grain Se concentrations. This led to increased human and animal Se intake and significantly decreased Se deficiencies among the population (Alfthan et al. 2015).

  Nevertheless, extensive research needs to be done to assess the relationship between micronutrient fertilization of crops and the nutrition and health status of people who consume these crops (de Valença et al. 2017).
- Spreading awareness among farmers about agronomic biofortification.
  In most developing countries where the problem of hidden hunger is prevalent, the farmers are resource-poor and they mostly follow the traditional agricultural practices. So, it is crucial to spread awareness among them about micronutrient fertilization advantages with respect to yield enhancement and health benefits. Moreover, agronomic biofortification strategies, which are being developed, should be region-specific and compatible with farmers' socio-economic conditions.

5 Conclusion

Different agronomic approaches for biofortification of staple food crops are mostly part of the innovative modern crop-raising practices which need to be promoted by the policymakers and planners and adopted by farmers. These cutting-edge approaches are the easiest, fastest, and affordable way to enrich their dietary intake with micronutrients by small and marginal farmers in developing Asian and African countries. In the fertilization approach, the foliar application performs better than soil application in terms of grain Fe and Zn enhancement in staple crops like rice, wheat, maize, and chickpea. Again, it requires a lesser amount of fertilizers. Seed priming or coating onto seeds with Zn has not been able to increase Zn concentration in grain significantly in most of the cereal crops except oats. However, it has several other benefits on seed health and soil-borne disease-pest suppression. The other approaches like microbial inoculation of PGPR and AMF, intercropping of dicot plant with a graminaceous plant, soil incorporation of short-duration green manure crops and irrigation are also useful in improving mobilization, uptake, and re-translocation of micronutrient from source to sink (grains). However, the agronomic biofortification approaches are facing several challenges, such as the meagre information and issues related to micronutrient bioavailability in the agronomically biofortified food grains and their impact on human nutrition. The other factors, viz. cost-effectiveness, determination of the optimum dose of application to raise a desirable level of mineral micronutrients in the plants' economic parts is other primary concerns for its acceptance. These challenges need to be addressed scientifically through coordinated efforts by researchers of various biological disciplines in tandem, along with policymakers and those involved in the public distribution systems.

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Micronutrients: Soil to Seed

20

Neha Gupta, Hari Ram, and Ismail Cakmak

Abstract

Plants are the main dietary resource of micronutrients essential for humans. But staple food crops on which people of poor and developing countries feed, do not contain sufficient micronutrient metals and thus results in poor growth, mental disorders and increased mortality of human consumers. Knowledge on mechanism of micronutrient uptake by plant roots, their accumulation in subcellular compartments, long-distance transport in vascular tissues, allocation to economic sinks of crop plant, etc. is, thus, of utmost importance in the biofortification programmes, implemented for nutrient enrichment of plant foods. The present chapter gives an insight on various physiological aspects regulating micronutrient absorption in crop plants. Despite the role of edaphic factors controlling micronutrient availability in soil solution, biological activity of root organs is also determinant of micronutrient metal uptake from rhizosphere. The charged nature of essential micronutrients, which are mostly divalent cations, needs sophisticated transporters for their delivery to respective sinks. Further to achieve metal homeostasis and to reduce their toxicity, root to shoot and shoot to root signalling is in concordance with metal chelators and transporters, which have been discussed in detail in present chapter. Finally, future research avenues have been discussed which can be targeted to enhance the efficacy of crop biofortification.

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Micronutrient · Biofortification · Metal transporters · Phloem translocation

1 Introduction

Plants are one of the fascinating sessile natural creatures that make aerobic life possible on planet earth. They possess unique property of preparing organic food from simpler inorganic substances, i.e. autotrophic nutrition. The inorganic nutrients which plants use to maintain their proper growth and development are classified in two types:

1. Macronutrients: These mineral nutrients are present in higher concentrations (i.e. >100 mg/kg DW) in plant parts.
2. Micronutrients: These mineral nutrients are present in low concentrations (i.e. <100 mg/kg DW) in plant parts.

Out of the 14 essential nutrient elements required for maintaining vegetative and reproductive growth of plants, 8 mineral elements (i.e. boron, chlorine, manganese, iron, nickel, copper, zinc and molybdenum) are micronutrients. These micronutrients are involved in primary and secondary metabolism of plant as enzyme cofactors, osmolytes, redox carriers in organic metabolites, signal transducing messengers, structural components of cell walls/membranes, regulators of hormone biosynthesis in addition to role in energy production and defence. The specific role of each of the micronutrients is enlisted in Table 20.1. In addition to essential elements, several other elements (such as selenium (Se), iodine (I), silicon, etc.) have been reported in specific plant taxa at low concentrations (i.e. <100 mg/kg DW). But such elements are not critical to all plants (Kaur et al. 2016). So, these are considered in category of beneficial elements instead of concentration similar to micronutrients. The present chapter will also briefly discuss two of such beneficial elements Se and I, which are well known in biofortification programmes for their crucial importance to humans.

1.1 Essentiality of Micronutrients for Plants and Animals

Plants are dietary resource for all 25 essential nutrients required for human growth. But low micronutrient density in edible crops is a serious global concern from the last two decades. Consumption of such low micronutrient food products leads to deficiency disorders in large fraction of humans consuming them. About two third of the world population suffers from 'micronutrient malnutrition' especially iron (Fe) and zinc (Zn) (White and Broadley 2009; Stein 2010). Despite of Fe/Zn, their deficiency leads to impaired physical activity, stunted growth, mental retardness and pregnancy issues (stillbirths and child deaths) (Stein et al. 2005). Fe deficiency (FeD)

Table 20.1 Role of various essential micronutrients in plants

Micronutrient (symbol)	Ionic form in Rhizosphere/ soil	Ionic form for root uptake	Cofactor in enzymes	Part of metabolite/metabolic process	References
Boron (B)	Boric acid or borate $B(OH)_3$	$B(OH)_3$		Cross linked with pectins, polyhydroxyl polymers—cell wall synthesis and stability Plasma membrane integrity Cell division, cytoskeletal polymerization Phenol metabolism Ammonium and nitrogen assimilation	Ahmad et al. (2009) Shireen et al. (2018)
Chlorine (Cl)	Cl^-	Cl^-	Asparagine synthetase Tonoplast V-type H^+-ATPase	 Facilitation of proton flux in PSII Osmoregulation Stabilization of membrane potential and pH gradients	Rognes (1980) Churchill and Sze (1984) Zhang et al. (2014b) White and Broadley (2001) Raven (2017)
Manganese (Mn)	Mn^{2+}, Mn_2O_3, MnO_2	Mn^{2+}	 Arginase Mn SOD Oxalate oxidase Phenylalanine ammonia lyase Phosphoenolpyruvate carboxylase (Mn/Mg)	Oxygen evolving complex of PSII in Photosynthesis Purine and urea catabolism ROS detoxification Pathogen defence Lignin biosynthesis—oxidation of monolignols by Mn^{3+} Tricarboxylic acid cycle-respiration	Alejandro et al. (2020), Bricker et al. (2012) Cao et al. (2010) Ravet and Pilon (2013) Requena and Bornemann (1999) Onnerud et al. (2002), Engelsma (1972) Gregory et al. (2009)

(continued)

Table 20.1 (continued)

Micronutrient (symbol)	Ionic form in Rhizosphere/ soil	Ionic form for root uptake	Cofactor in enzymes	Part of metabolite/metabolic process	References
Iron (Fe)	Fe^{2+}, Fe^{3+}	Fe^{2+}	Fe-SOD, catalase	ROS scavenging	Ravet and Pilon (2013)
			Nitrate reductase Nitrite reductase Glutamate synthetase	Nitrogen assimilation	Balk and Lobreaux (2005)
			APS reductase Sulphite reductase	Sulphate assimilation	Balk and Lobreaux (2005)
			ACC oxidase	Ethylene biosynthesis	Pech et al. (2003)
			DNA polymerase, DNA helicase and primase	DNA replication and repair	Zhang (2014)
				Cytochrome- photosynthesis and respiration Fe-S cluster in Psa A, Psa B, Psa C of PSI-photosynthesis Ferredoxin in redox homeostasis	Ravet and Pilon (2013)
Nickel (Ni)	Ni^{2+}	Ni^{2+}		Homeostasis of glutathione	Noctor et al. (2012)
			SOD	ROS scavenging	Mustafiz et al. (2014)
			Glyoxalases (family I)	Detoxification of reactive carbonyl species	Sousa Silva et al. (2013)
			Ureases	Nitrogen assimilation	Sirko and Brodzik (2000)
			Methyl-CoM reductase	Biological methanogenesis	Su et al. (2019)
			Peptide deformylases	Protein processing in chloroplasts	Hanson et al. (2000)
			Some hydrogenases	Biological N fixation	Brito et al. (1994)

Copper (Cu)	Cu^{+}, Cu^{2+}	Cu^{+}	Cu Zn-SOD Cytochrome c oxidase Laccase Amine oxidase Polyphenol oxidases	ROS scavenging Cytochrome complex-ETC of respiration lignin production Oxidation of polyamines Quinone biosynthesis from diphenols (plant defence) Plastocyanin in ETC of photosynthesis Plantacyanins	Ravet and Pilon (2013) Berthet et al. (2012) Puig (2014) Sullivan (2015) Ravet and Pilon (2013) Feng et al. (2013)
Zinc (Zn)	Zn^{2+}	Zn^{2+}	Zn^{2+}—cofactor for >300 enzymes Cu/Zn—SOD Carbonic anhydrase Alkaline phosphatase Alcohol dehydrogenase	 ROS scavenging Hydration of CO_2 to release bicarbonate Hydrolysis of phosphate esters Anaerobic respiration/alcoholic fermentation Tryptophan biosynthesis (auxin precursor) Zn, finger proteins; regulation of gene expression Metabolism of carbohydrates, lipids and nucleic acids	 Broadley et al. (2007), Gupta et al. (2016) Englbrecht et al. (2004) Palmer and Guerinot (2009)
Molybdenum (Mo)	MoO_4^{2-}	MoO_4^{2-}	Sulphite oxidase Aldehyde oxidase Xanthine oxidoreductase Nitrate reductase Amidoxime-reducing component (ARC)	Sulphite detoxification in to sulphate ABA biosynthesis Purine metabolism Nitrate assimilation Reduction of nitrite to NO	Brychkova et al. (2015) Verma et al. (2016) Maia and Moura (2011) Chamizo-Ampudia et al. (2017) Yang et al. 2015, Chamizo-Ampudia et al. (2016)

ACC 1-aminocyclopropane-1-carboxylic acid; *ETC* electron transport chain; *PSI* and *PSII* photosystem I and II; *ROS* reactive oxygen species; *SOD* superoxide dismutase

mainly results in anaemia while Zn deficiency (ZnD) often leads to diarrhoea and pneumonia in infants and adults. Zn deficiency also leads to hypogonadism, immune dysfunction, DNA damage and cancer development (Gibson 2006; Prasad 2009). Thus, 'hidden hunger' caused due to micronutrient deficiency is one of the serious global issues. This problem is aggravated in developing countries where cereal-based products are main staple foods that contain antinutritional substances (such as phytate, tannic acid) and possessed low micronutrient density and bioavailability. More than two billion of world population suffers from micronutrient deficiency with approximately 0.8 million deaths are reported annually. In addition to FeD and ZnD, deficiency of Se (SeD) and I also associated with severe health consequences; with about 15 and 30% of world population lacks these nutrients. Iodine deficiency (ID) impairs thyroxin production causing goitre, irreversible mental retardation (autism), reproductive dysfunction and cretinism under severe deficiency (de Benoist et al. 2008). SeD leads to dermatitis, hair loss and garlicky breath, male infertility and increased incidence of cancers, respiratory failure, myocardial infarction and renal failure under severe selenosis (Fordyce 2013). These dietary mineral intakes are of extreme importance to pregnant women, where deficiency of any above micronutrients (Fe, Zn, I and Se) can lead to irreversible brain damage, permanent foetal developmental disorders and cognitive decline in developing foetus (Fordyce 2013; World Health Organization 2007).

1.2 Micronutrients Involved in Biofortification Programmes for Crop Improvement

Almost one in three people worldwide suffers from micronutrient malnutrition (FAO, IFAD, WFP 2015). Thus, to sustain good health and development, of individuals having limited access to diverse diets, various interventions such as supplementation, industrial food fortification, etc. have been put forward. But among them, the most promising and cost-effective approach is biofortification. This biological process of nutrient enrichment utilizes tools of conventional plant breeding, agronomic management methods and techniques of transgenics to enhance micronutrient density of staple food crops. Due to crucial importance of Fe, Zn, Se and I for human growth and their widespread deficiency, these four nutrients are of main focus in biofortification. The international programme of Harvest Plus addresses a substantial number of researches on biofortification to develop biofortified iron crops, zinc crops, iodine crops, vitamin A crops, etc. in cereals (wheat, rice, maize, barley, pearl millet), pulses (soybeans, common bean, lupines) vegetables (cassava, orange sweet potato, carrot, cauliflower, potato, tomato) and fruits (papaya, banana, etc.) (Bouis and Saltzman 2017). The two main approaches used in biofortification are genetic biofortification and agronomic biofortification.

The genetic biofortification (GB) enhances plant's own inherent potential (genetic potential) of nutrient acquisition from soil. The rich gene pool of wild germplasm provides candidate genomic segments for introgression in cultivated varieties in genetic biofortification to enhance root uptake of micronutrients, their

remobilization to edible sinks and even to maintain metal homeostasis. But if soil is itself nutrient poor, in that case soil and foliar application of nutrient fertilizers is used for nutrient enrichment of crops grown as in case of agronomic biofortification. This approach is also a shotgun approach in those crops where loss/lack of natural wild diversity occurs.

2 Soil as Reservoir of Micronutrients

It is well known that soil is the main reservoir of mineral elements, for crop plants. The widespread deficiencies of micronutrients in cultivated soils limit not only micronutrients concentration in crop produced but also affected crop yields. Various edaphic factors contribute significantly towards nutrient concentrations in rhizosphere where plant roots communicate with soil environment to favour mineral absorption even under nutrient-deficient conditions (Fig. 20.1). Some of them are given below.

2.1 Factors Affecting Availability of Micronutrients in Soil Solution

2.1.1 Soil Formation

As soil is formed from weathering of rocks, the rocks (e.g. igneous rocks) rich in micronutrients will lead to formation of soils with high concentration of micronutrients. Further, the process of soil formation from parent rock material is another factor which influences micronutrient contents of soil. The non-uniform distribution of micronutrients in different geographical zones is due to differences in parent rock materials and processes that lead to formation of soil. The soil formed after weathering has higher concentration of micronutrients than parent rock due to low mobility of these divalent cations.

2.2 Soil Moisture and Texture

Soil moisture and texture affects micronutrient concentration in soil solution through diffusion/mass flow. Coarse-textured sandy soils exhibit enhanced leaching of available micronutrients more than dry loam soils.

2.3 Soil pH

Another determining factor which affects nutrient solubility in soil solution is soil pH. High pH in alkaline/saline soil favours formation of less available micronutrient (particularly Zn, Mn, Fe, etc.) organic complexes and results in their deficiency. In contrast, acidic pH results in decreased adsorption of cationic nutrients to cation

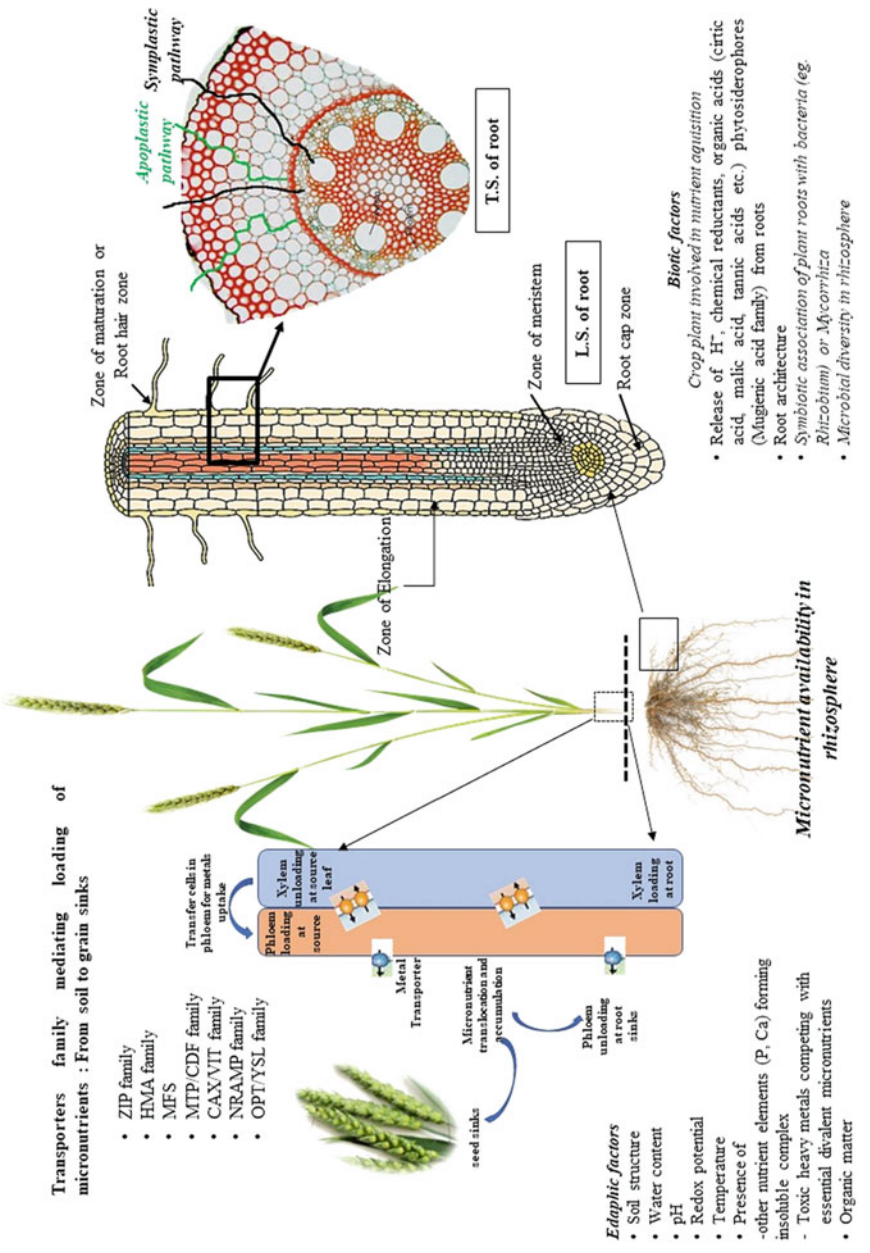

Fig. 20.1 An overview of micronutrient absorption in plants

exchange sites of soil constituents (such as clay mineral, metal oxides) and makes them available in soil solution. A unit increase in soil pH is found to decline Zn availability by 30- to 45-fold at a pH range of 5.5–7.5. Tight adsorption of Fe^{3+} or Zn^{2+} as metal oxides, phosphates or carbonates also makes these micronutrients unavailable in rhizosphere for uptake by roots.

2.4 Soil Organic Matter

Organic matter is the source of numerous soluble organic compounds which regulate microbial activity in rhizosphere. The wide diversity of microorganisms in soil is responsible for release of various organic acids, chelator compounds, etc. that favours free release of micronutrient cations in soil solution.

2.5 Others

In addition to above, redox potential of soil, the presence of similar charged toxic divalent cations (such as Pb^{2+}, Cd^{2+}, As^{2+}, Hg^{2+}), microbiota, etc. also alter mineralization and hence phytoavailability of micronutrients in rhizosphere.

3 Absorption of Micronutrients by Plant as Biological System: Uptake, Transport and Sequestration

In order to enrich food crops with tools of biofortification, there is a need of deep understanding of the micronutrient acquisition, their xylem and phloem transport, sequestration and translocation to edible plant sinks. As most of the essential micronutrients belong to category of heavy metals, there should be a tight regulation in their uptake and metabolism by plant system; otherwise, these will lead to oxidative stress and will be toxic to cellular machinery. Plants maintain metal homeostasis from cellular level to whole plant level in hierarchy through specialized transporters, chelating ligands and sequestration of toxic metal ions in intercellular compartments. These will be discussed in detail below:

3.1 Roots-Organs Mediating Nutrient Uptake from Rhizosphere

Plant roots are specialized organs which mediate nutrient absorption from soil solution. The unicellular hairs present on root epidermis perform dual function in nutrient acquisition i.e. (1) enhancing phytoavailability of micronutrients in rhizosphere and (2) subsequent transport of micronutrients through transporters/channel proteins to inner root cells for xylem loading. Roots are not just static organs in rhizosphere; their continuous activity such as release of exudates and mucilage in soil prevents damage to growing apical meristem and allow tight binding of lateral

roots to soil substratum. The activity of root hair cell plasma membrane (RCPM) H^+-ATPase mediates extracellular acidification in rhizosphere which helps in active uptake of charged micronutrients by declining soil pH. The release of organic acids (such as citric acid, tannic acid, oxalic acid, tartaric acid) in mucilage and exudates of damaged root cap cells also increase solubility of micronutrients by maintaining low soil pH.

The soluble metal cations enter in cytosolic compartment of root hair cell either through transporters/channel proteins. This metal ion uptake can be passive (i.e. along the concentration gradient from higher metal ion concentration in soil solution towards low concentration present in root hair cell) or active (against the concentration gradient) depending upon metabolic essentiality of that metal ion. It is very important to mention here that a wide diversity in uptake mechanism exists among plants for the different metal cations and even for same metal atom with different available forms. For example, Fe existed in Fe^{2+} form with predominance of Fe^{3+} in rhizosphere. But plant iron transporters allow uptake of Fe^{2+} ion. Thus, two specialized mechanisms existed in higher plants to mediate Fe uptake:

1. Strategy I: Reduction of Fe^{3+}in to $Fe^{2+}$by ferric-chelate reductase (Robinson et al. 1999) encoded by FRO gene family. Such reduction mechanism is found in non-graminaceous monocots and dicot plants. These Fe^{2+} ions are then entered in root cell through iron transporters.
2. Strategy II: Release of phytosiderophores (metal chelators) such as mugineic acids, avenic acid, distichonic acid, etc. to bind with Fe^{3+} followed by uptake of Fe (III)-ligand chelating complex by specific yellow stripe 1 (YS1) or YS1-like (YSL) transporters. A wide range of phytosiderophores are secreted from cereal family (graminaceous monocots) which play crucial role in metal uptake under Fe/Zn deficiency. Further, amounts of these phytosiderophores released in rhizosphere, determine the tolerance of particular plant species to soils with limited Fe or Zn phytoavailability. For example, roots of barley and wheat secrete large amounts of mugineic acid compounds (MAs) than rice and, thus, confer enhanced tolerance to Fe-limiting soils. Moreover, barley root secretes a range of MA species including mugineic acid (MA), 3-epihydroxymugineic acid, 3-epihydroxy-2′-deoxymugineic acid and 2′-deoxymugineic acid (DMA) than DMA alone by bread wheat, thus, increased tolerance potential of barley to Fe-limited environments (Romheld and Marschner 1990).
3. Combination of both strategy I and strategy II as occur in rice.

3.2 Root Uptake of Mineral Nutrients Zn, Se and I

Zn is mainly absorbed in Zn^{2+} form. Absorption of this divalent cation by root hair cell interior would cause depolarization of plasma membrane and will reduce its further transport. Thus, to maintain Zn^{2+} influx, RCPM H^+-ATPase cause efflux of H^+, resulting in hyperpolarization of RCPM which acts as driving force for Zn uptake by plant roots. Zn^{2+} ions are mainly transported through specific ZIP family

transporters. However, Zn-chelating complexes formed due to binding of metal chelators (such as MAs, nicotianamine, histidine, etc.) with Zn^{2+} ions, are transported through specific YSL transporters in cytoplasmic interior of root cell. In contrast to Zn^{2+} and Fe^{2+}, Se and I are absorbed as anions. Natural Se exists in various forms as selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), selenide (Se^{2-}), element Se (Se^0) and organoselenium compounds (selenocysteine (Se Cys) and selenomethionine (Se Met)) in rhizosphere. But plant roots are able to take up only selenate, selenite, SeCys and Se Met from soil solution. Out of all phytoavailable forms, SeO_4^{2-} is absorbed readily from soil solution than SeO_3^{2-}. Predominance of Se form in soil solution depends upon concentration, pH of soil, redox potential, organic matter, presence of other nutrients particularly sulphur, iron oxides, etc. (Sors et al. 2005). Generally, SeO_4^{2-} is mainly present in alkaline soils, while well-drained acidic to neutral soils contain SeO_3^{2-}. Due to the presence of negative charge on root cell membrane, SeO_4^{2-} and organoselenium compounds are absorbed actively through H^+/anion symporter, anion channels and amino acid transporters present on RCPM. The sulphate transporters (both high- and low-affinity transporter) present on RCPM mediate SeO_4^{2-} uptake in addition to sulphate. This active uptake involves cotransport of three protons for each SeO_4^{2-} ion. However, organoselenium compounds are transported through amino acid transporters similar to cysteine and methionine. Selenite is found to be transported passively through phosphate transporters (Li et al. 2008).

The beneficial nutrient iodine is taken up both in inorganic forms as iodide (I^-) and iodate (IO_3^-) and organic iodine by plant roots. Till date no iodine transporters have been discovered in plants. But it has been reported that roots cells have iodate reductase/specific nitrate reductases which convert IO_3^- in to I^- anion, due to high solubility of iodide than iodate. I^- anions are effectively absorbed by plant roots (Whitehead 1973) but are susceptible to leaching. These I^- anions are taken up through chloride channels and further loaded in to xylem through specific anion channels (Blasco et al. 2008; Caffagni et al. 2011; Roberts 2006). Thus, energized transport of micronutrients along with Se and I is under tight metabolic control of transporter proteins. Plants also exhibits an inherent potential to cope the micronutrient deficiency through various mechanisms such as (a) oriented root growth and enhanced lateral branching (b) increased root exudation (c) overexpression of specific high-/low-affinity root membrane transporters (d) release of specific micronutrients from subcellular stores (e) remobilization of micronutrient from senescing tissue, etc. to fulfil the demands for plant growth and metabolism.

3.3 Apoplastic and Symplastic Routes in Radial Transport of Nutrients

Micronutrients once acquired by root hair cells can traverse through symplastic (intracellular) or apoplastic (extracellular) pathway for their loading in to xylem (Fig. 20.1). However, suberin deposits on endodermis (i.e. Casparian strips) is the

major barrier to apoplastic transport. This water impermeable layer allows only symplastic transport of charged nutrients from cortex to endodermis. But after this check barrier, nutrient transport can again be apoplastic/symplastic to xylem. The radial transport of nutrients towards root stele increases nutrient concentration in subsequent inner tissue layers due to decreased radii. In this specialized transport, micronutrient sequestration also takes place in different subcellular compartments. Due to charged nature of micronutrients (especially Fe, Zn, Mn, Ni, etc.), these divalent metal cations bind to low molecular weight chelators for facilitating their symplastic transport and intracellular sequestration. A large number of molecules such as organic acids (e.g. citrate, malate), histidine, phytate, mugineic acid, nicotianamine, etc. have been implicated as important cellular ligands for cytosolic and vacuolar sequestration of Zn, Fe, Cd, Ni, etc. (Haydon and Cobbett 2007a; Ma et al. 2005). Such metal ligands not only help in maintenance of cellular homeostasis but also contribute to metal translocation towards storage sinks. Once loaded in xylem, these micronutrients reach to aerial shoots under the influence of transpiration pull and mass flow. The whole process of nutrient uptake and subsequent transport to shoot is dependent upon developmental stage and environmental factors which regulate expression of genetic components mediating nutrient absorption.

After reaching to aerial leaves, xylem unloading and phloem loading of nutrient initiates which is crucial for root to shoot and shoot to root mineral cycling. In phloem tissue, transfer cells play a crucial role in energized loading of micronutrients and thus channelizes them to developing sinks for accumulation. Further, for metal accumulation in aerial tissues, a wide variation of cellular ligands exists in different species for same metal cation. For example, *T. caerulescens* utilizes citric acid, while *A. halleri* utilizes malic acid for Zn storage in vacuole (Kupper et al. 2004; Ma et al. 2005). Zn–His complex is generally formed to chelate free Zn^{2+} ions in cytosol (Kupper et al. 2004).

In case of beneficial elements such as iodine, xylem transport predominates over phloem transport (Weng et al. 2008) resulting in its less accumulation in sink tissues with undeveloped xylem. Absorption of Se is similar to sulphate, where enzymes of sulphur assimilation pathway convert SeO_4^{2-}in to Se Cys in chloroplast which is then further converted in Se Met in cell cytosol. However, in Se hyperaccumulators, selenocysteine is converted into non-protein amino acids like Se-methyl selenocysteine, γ-glutamyl-Se-methylselenocysteine and selenocystathionine for Se detoxification (White 2018).

3.4 Transporters and Channel Proteins Involved in Micronutrient Transport

The selective uptake of micronutrients from rhizosphere, their subsequent loading in xylem, storage in intracellular compartments and further translocation to seed/grain sinks in a plant system is only possible due to the presence of specialized membrane transporters which facilitate movement of transition metal ion/micronutrients both intracellularly and intercellularly. Such transporter proteins can be either substrate

specific or may bind to different metal cations with differential affinity. But one thing is sure that the presence of membrane transporters allows selective inflow-outflow of the charged micronutrients. A large number of transporter proteins families such as ZIP family, HMA family, MFS family, MTP family, YSL family, VIT family, CAX family, etc. have been discovered which are involved in regulation of micronutrients (Fe, Zn, Mn, Co, etc.) transport, their accumulation and detoxification under excess, to maintain metal homeostasis (Table 20.2). A few of them are discussed in detail here.

3.5 ZR- and IRT-Like Proteins (ZIP) Family

This transporter family gets its name from the first members identified, i.e. zinc-regulated transporter (ZRT) in yeast and iron-regulated transporter (IRT) like proteins identified in *A. thaliana* which mediate influx of Zn^{2+} and Fe^{2+} ions in to the cytoplasm. Apart from these micronutrients, some of ZIP transporters have also been shown to transport Mn^{2+}, Cu^{2+} and Cd^{2+} ions. Most of the members of this family are present either on cell plasma membrane for micronutrient uptake or on integral membranes of intracellular compartments such tonoplast, endoplasmic reticulum, etc. for nutrient translocation to various plant organs (Ajeesh Krishna et al. 2020). Topological studies reveal that ZIP proteins have characteristic eight transmembrane domains (TMD) with their N- and C-termini towards extracellular space. These transporter proteins vary in length from 309 to 476 amino acids. This difference in length of ZIP proteins is due to varied length of potential metal binding domain between TMD III and IV (Guerinot 2000). The conserved sequence present in TMD IV of ZIP members contains histidine residues which play a crucial role in metal transport.

Various orthologs of ZIP proteins have been identified in many crop species such as 12 in barley (Tiong et al. 2014), 14 in wheat (Evens et al. 2017), 17 in rice (Chen et al. 2008) and 23 in common bean (Astudillo et al. 2013). These orthologs differ in their cellular localization, tissue-specific expression, substrate specificity and catalytic potential of metal transport. Among the 15 ZIP family members of *Arabidopsis* (Milner et al. 2013), AtIRT1 is the most well studied for Fe uptake and transport. Recently, Milner et al. (2013) characterized functional ability of 11 At ZIPs in metal transport and revealed involvement of AtZIP1 and AtZIP2 in root to shoot translocation of Zn and Mn. Out of 11 transporters, 6 (ZIP1, ZIP2, ZIP3, ZIP7, ZIP11 and ZIP12) members complement Zn transport, six (ZIP1, ZIP2, ZIP5–7 and ZIP9) mediate Mn, and one (ZIP7) complements Fe transport in yeast mutants. Further, the role of ZAP1 transcriptional factors in transcriptional and post-transcriptional regulation of ZRT1 and ZRT2 has been demonstrated in yeast (Zhao et al. 1998). These transcription factors bind to a zinc-responsive element (ZRE) present in promoter region of ZRT genes to control their expression.

The presence of such zinc deficiency response elements (ZDREs) has also been reported in plant ZIP family such as AtZIP1, AtZIP3, AtZIP4, AtZIP5, AtZIP9 and AtZIP10 (Assuncao et al. 2010) where binding of bZIP (basic leucine zipper

Table 20.2 Channels and transporter proteins involved in uptake and transport of micronutrients/beneficial elements

Micronutrient	Ionic form for root uptake	Concentration in plants ($\mu g\ g^{-1}$ DW)	Channels and transporter proteins mediating long-distance transport	References
B	$B(OH)_3$	20–100	Major intrinsic proteins: AtNIP 5.1, AtNIP 6.1, OsNIP3.1 High-affinity active transport: BOR1, BOR2	Takano et al. (2006), Tanaka et al. (2008), Hanaoka et al. (2014) Nakagawa et al. (2007), Takada et al. (2014)
Cl	Cl^-	1–20	CLCs family: At CLCa, AtCLCc, At CLCg CCC family: AtCCC, OsCCC1 CPA2 family: GmSALT3/CHX1 ALMT family: AtALMT9, AtALMT12 SLAC/SLAH family: AtSLAC1, AtSLAH1 NRT/NPF family: AtNPF2.4 and AtNRT1.5	Jossier et al. (2010), Nguyen et al. (2016) Colmenero-Flores et al. (2007), Kong et al. (2011) Liu et al. (2016) De Angeli et al. (2013), Meyer et al. (2010) Negi et al. (2008) Li et al. (2017), Lin et al. (2008)
Mn	Mn^{2+}	30–500	CaCA family: At CAX2, At CAX4, AtCAX5 NRAMP family: At NRAMP1–4, Ah NRAMP1 YSL family: Os YSL2, Os YSL6 ZIP family: At ZIP1–2, At ZIP 5–9 CDF/MTP family: At MTP8–11	Edmond et al. (2009), Mei et al. (2009) Thomine et al. (2000), Lanquar et al. (2010) Yang et al. (2014), Sasaki et al. (2011) Milner et al. (2013) Delhaize et al. (2007)
Fe	Fe^{2+}	50–100	NRAMP family: AtNRAMP3 and AtNRAMP4 OPT family: AtYSL3, OsYSL2, OsYSL15 ZIP family: At IRT1–3 MATE family: FRD3, FRO2 VIT family: At VIT1 MFS family: IREG1–3	Lanquar et al. (2005) Waters et al. (2006), Ishimaru et al. (2010) Korshunova et al. (1999) Rogers and Guerinot (2002), Connolly et al. (2003) Kim et al. (2006) Nino Gonzalez et al. (2019)
Ni	Ni^{2+}	0.05–10	NRAMP family: Ah NRAMP1 YSL/OPT family: TcOPT3, Zm YS1, At YSL2 ZIP family: At IRT1–3, At ZIP1–6, At ZIP9–10	Wang et al. (2019) Hu et al. (2012), DiDonato Jr et al. (2004), Korshunova et al. (1999), Pedas and Husted (2009)
Zn	Zn^{2+}	25–150		
Cu	Cu^+, Cu^{2+}	5–20	COPT family: At COPT1–6 HMA family: AtHMA5–8, Os HMA5 ZIP family: ZIP2 and ZIP4	Puig (2014) Blaby-Haas et al. (2014), Deng et al. (2013) Wu et al. (2015)

(continued)

Table 20.2 (continued)

Micronutrient	Ionic form for root uptake	Concentration in plants (μg g^{-1} DW)	Channels and transporter proteins mediating long-distance transport	References
			H^+/Cu^{2+} antiporters YSL family: At YSL1–3, Os YSL16 MFS transporter: TaCT1	Parrotta et al. (2015) Chen et al. (2011), Lee et al. (2012) Li et al. (2014)
Mo	MoO_4^{2-}	0.1–1	MOT1 family: AtMOT1, AtMOT1.2 MOT2 family: CrMOT2 Sulphate transporters: SHST1	Gasber et al. (2011) Tejada-Jimenez et al. (2011) Fitzpatrick et al. (2008)
Beneficial elements				
Se	SeO_4^{2-}, selenite (SeO_3^{2-}, $HSeO_3^-$) and organoselenium compounds (Se Cys and Se Met)		SULTR transporter: At SULTR1;1, At SULTR1;2 Phosphate transporter: OsPT2, Os PT8	Shibagaki et al. (2002), Rouached et al. (2008) Zhang et al. (2014a), Song et al. (2017)
I	IO_3^-, I^-		CLC family Voltage gated R and S-type anion channels—iodine transporter not yet discovered	Gonzali et al. (2017), Roberts (2006)

ALMT aluminium-activated malate transporters; *CaCA* calcium/cation antiporter; *CCCs* cation/chloride cotransporters; *CDF* cation diffusion facilitator; *CHX* cation/H+ exchanger; *CLC* chloride channels; *COPT* copper transporters; *CPA2* cation proton antiporter 2; *FRD* ferric chelate reductase defective; *FRO* ferric reductase; *HMA* heavy metal ATPases; *MOT* molybdate transporters; *MATE* multidrug and toxic compound extrusion; *MFS* major facilitator superfamily; *MTP* metal tolerance protein; *NRAMP* natural resistance-associated macrophages protein; *NRT/NPF* nitrate transporter/nitrate peptide transporter; *OPT* oligopeptide transporter; *SLAC/SLAH* slow-type anion channel/associated homolog; *SULTR* sulphate transporter; *VIT* vacuolar iron transporter; *YSL* yellow stripe 1-like; *ZIP* ZRT- and IRT-like proteins

domain) transcription factors, i.e. bZIP19 and bZIP23, enhances the expression of ZIP members under Zn deficiency. But how these bZIP transcription factors sense low cellular Zn^{2+} concentration is still to be elucidated. Regarding their functioning, Assuncao et al. (2013) suggested that Zn^{2+} ion binds to cysteine-histidine-rich motif of bZIP transcription factor dimers under normal cellular Zn conditions and causes its inactivation. But under low Zn, active bZIP dimer binds to ZDRE motif of ZIPs promoter and results in their increased transcription for enhancing Zn uptake. In addition to Zn, many ZIP members also transport other divalent metal cations (such as Cd^{2+}, Cu^{2+}, etc.) which are toxic to plants (Tan et al. 2020). Recently, overexpression of OsZIP1 in transgenic rice was found to reduce Zn, Cu and Cd accumulation under excess metal (Liu et al. 2019). Thus tight regulation of ZIP members is essential to maintain metal homeostasis.

3.6 Heavy Metal ATPases (HMA) Family

The members of HMA family mediate heavy metal transport across the biological membranes by utilizing ATP as energy source. This family is also known as P_{1B}-ATPase family. Depending upon their metal specificity, HMA family is divided in two subgroups: (1) a Cu/silver (Ag) group and (2) a Zn/Co/Cd/lead (Pb) group (Takahashi et al. 2012). HMAs are basically efflux transporters that are ubiquitously present in archaea, prokaryotes and eukaryotes including plants. These transporters play a key role in transition metal detoxification. Plant HMA proteins have basic structure of eight transmembrane helices (TM) with their N- and C-terminal ends towards cytosol. There is the presence of two cytoplasmic loops, i.e. one small between TM 4 and TM5 and other large between TM 6 and TM7. Each member of HMA protein possesses three functionally important domains which are conserved across all P-type ATPases, i.e. cytoplasmic actuator (A) domain located in smaller loop, phosphorylation (P) domain and nucleotide (N) domain present in large cytoplasmic loop responsible for ATP binding. In addition, a specific CPx motif present in TM 6 of all P_{1B}-ATPase, is involved in metal translocation. Some putative metal-binding domains (MBD) are present in the N- or C-terminal regions of HMA proteins (Williams and Mills 2005; Arguello et al. 2007). The MBD of N-terminus contains a highly conserved CxxC residue in HMA domain which controls turnover rate of P_{1B}-ATPase. The presence of histidine- and cysteine-rich region occurs in MBD of C-terminus which plays a role in metal selectivity of this transporter protein (Lutsenko et al. 2003; Mandal and Arguello 2003).

The members of HMA family are highly diverse in terms of their tissue distribution, subcellular localization, metal specificity and regulation. For instance, *Arabidopsis* contains eight HMA transporters (AtHMA1–8), in which AtHMA1–4 carry out transport of transition metals Zn^{2+}, Cd^{2+}, etc. (Mills et al. 2005; Eren and Arguello 2004). AtHMA5–8 belongs to subgroup II and is involved in delivery of Cu to chloroplast proteins of thylakoid lumen and stroma.

AtHMA2 and AtHMA4 express in vascular tissues of root, stem and leaves and mediate long-distance transport of Zn. Expression of AtHMA3 on tonoplast results

in sequestration of Zn^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+} ions for detoxification. AtHMA1 is involved in detoxification of excess Zn in chloroplast (Kim et al. 2009). The HMA2 homologues are highly conserved in Poaceae as both OsHMA2 in rice and HvHMA2 in barley are functionally similar in carrying root to shoot transport of Zn and Cd (Mills et al. 2012). So, understanding of regulatory networks controlling HMA transporters will provide opportunities to enhance micronutrient levels in biofortified crop with minimized risk of toxic metals in edible sinks.

3.7 Major Facilitator Superfamily (MFS)

This superfamily consists of large group of secondary active membrane transporter proteins that utilize electrochemical potential of proton transport as driving force to carry out import or export of small organic molecules including transition metals Zn^{2+} and Fe^{2+}. Most of the MFS proteins contain 12–14 transmembrane α-helices with a large, cytoplasmic loop between TMD6 and TMD7. A conserved MFS domain is also found between TMD2 and TMD3. The MFS members are ubiquitously present in all living organisms, but in plants ferroportin, drug-H^+ antiporter-1 (DHA1) and uncharacterized TET families have been discovered till date as metal transporters. A wide functional diversity and substrate specificity of these MFS transporters reveal their physiological significance in plants (Nino Gonzalez et al. 2019). The ferroportin family of *A. thaliana* includes member IREG1, IREG2 and IREG3 which are involved in efflux of Fe^{2+} across membrane (Morrissey et al. 2009). Members of DHA-1 family such as ZIF1 (zinc-induced facilitator 1) and ZIF-like 1(ZIFL1) confer increased tolerance to Zn by sequestering Zn^{2+}or Zn chelates in vacuole (Haydon and Cobbett 2007a, b).

In addition to above, some MFS transporters are involved in root uptake and phloem transport of divalent micronutrients (Zn^{2+}, Fe^{2+} Mn^{2+} and Cu^{2+})-NA/MAs complexes. TOM family, i.e. transporter of mugineic acid family phytosiderophores, is one such example from MFS, which is involved in efflux of DMA to cell exterior. Once released, DMA binds to metal cations (particularly Fe^{2+}) in soil solution and helps in its internal *in planta* transport. TOM1 in rice mediates Fe acquisition from rhizosphere. Another homologue TOM2 facilitates metal transport through plant body. TOM2 mediates efflux of DMA in apoplasm which chelates not only Fe^{2+} but also Zn^{2+} and Cu^{2+}. This functionality of TOM2 is demonstrated to enhance metal translocations to sinks under normal plant growth (Nozoye et al. 2015). In addition to TOM family, a recently characterized ENA1 (efflux transporter of NA) transporter maintains Fe homeostasis in rice. ENA1 is found to maintain intracellular trafficking of NA-metal complex for vacuolar detoxification of Fe (Nozoye et al. 2019). Another class of phenolics efflux zero 1 and 2 (PEZ1 and PEZ2) transporter proteins in MFS mediates efflux of protocatechuic acid. They are reported to contribute in long-distance transport of iron through root xylem (Ishimaru et al. 2011; Bashir et al. 2011).

3.8 Metal Tolerance Proteins (MTPs) Family

As their name suggests, these transporter proteins are involved in tolerance to micronutrient metals accumulated at toxic levels. Plant MTPs are also known as cation diffusion facilitator (CDF) family. Their main function is efflux of metal cations out of cytosol either in subcellular compartments or to extracellular space. Thus, the role of MTP members in heavy metal homeostasis, its detoxification and hyperaccumulation has been discovered so far in plants (Ricachenevsky et al. 2013). Among them, AtMTP1 and AtMTP3 are the most functionally characterized vacuolar transporters. They preferentially transport Zn^{2+} but also transport Ni^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+} and Fe^{2+} with varied affinity (Arrivault et al. 2006). Structurally plant MTPs possess six TMDs with their N- and C-termini towards cytosol. A conserved CDF signature exists between TMD 2 and 3 (Gustin et al. 2011). A histidine-rich cytoplasmic loop is also present between TMD 4 and 5. This cytoplasmic loop acts as metal sensor to determine its cytoplasmic levels and determines metal selectivity. MTPs function as H^+-metal cation antiporters with broader substrate affinity. These proteins are generally specified as Zn-CDFs, Fe/Zn CDFs and Mn-CDFs phylogenetically based upon substrate metal ion. But they are also able to transport other heavy metal divalent cations (Montanini et al. 2007). Anuradha et al. (2012) confirmed an increase in expression of OsMTP1 on exposure to metals such as Fe, Cu, Cd, Zn, etc. which accelerated metal accumulation in grain sinks.

3.9 CAX and VIT Family

Vacuolar sequestration of micronutrients is one of the mechanisms to maintain micronutrient homeostasis in cytosol and for their precise allocation to desired sinks. To fulfil this function, efflux transporters of CAX (cation exchanger) family and VIT (vacuolar iron transporter) family are present on tonoplast.

3.9.1 CAX Family

CAX are cation/H^+ antiporters which belong to Ca^{2+}/cation antiporter (CaCA) superfamily (Shigaki and Hirschi 2006). Phylogenetically, CAXs are grouped into three types with plant CAXs belong to category of Type I. Type I CAXs are further divided in two distinct groups—Type IA and Type IB. CAX proteins are encoded by a multigene family and have a structural characteristic of 11 TMDs (Shigaki et al. 2006). The TMDs are divided in three components as TMD1, TMD2–6 and TMD7–11. Among them, TMD1 contains a highly variant nine-amino-acid region which regulates metal cation specificity during transport. Both components TMD2–6 and TMD7–11 are thought to be formed with ancient duplication event. A highly conserved cation-binding region is present between TMD 2 and 3 and TMD7 and 8.

The presence of an N-terminal auto-inhibitory domain has also been detected in a range of plant CAXs. Numerous CAXs have been functionally characterized in *Arabidopsis*, barley, tomato and rice (Edmond et al. 2009; Kamiya et al. 2006). In

Arabidopsis, AtCAX2 and AtCAX4 are involved in Cd^{2+}, Mn^{2+} and Zn^{2+} detoxification under heavy metal stress, while AtCAX5 (ortholog of AtCAX2) regulates only Mn^{2+} transport (Korenkov et al. 2007; Zhang et al. 2011) under metal excess. Thus, a wide diversity is present among CAX orthologs for their functional characteristics and broad substrate specificity which alters regulatory mechanism of intracellular sequestration in vacuole.

3.9.2 VIT Family

VIT proteins mediate transport of ferrous ions into vacuoles and thus regulate Fe homeostasis in plants. These transporter proteins exhibit high homology to CCC-1 (Ca^{2+}-cross-complementer) protein of yeast that catalyses intracellular storage of Fe in vacuoles. The first member of VIT family identified *in planta* is AtVIT1 which is involved in Fe loading in seed. Various VIT-1like protein (VTL) transporters have been identified in *Arabidopsis*, rice, wheat, tulip, etc. which exhibit strong selectivity for Fe^{2+} but are also able to transport other metal cations, i.e. Zn^{2+} and Mn^{2+} (Kim et al. 2006; Gollhofer et al. 2014; Eroglu et al. 2017; Zhang et al. 2012; Sharma et al. 2020; Connorton et al. 2017; Momonoi et al. 2009). Differential tissue expressions of these VIT and VTL transporters in plants allow capturing of excess cytoplasmic Fe in vacuolar compartments of different sinks. While AtVIT1 has high expression in provascular tissues of wild-type embryo (Kim et al. 2006), OsVIT1 and OsVIT2 are expressed in flag leaves and regulate partitioning of Fe and Zn in developing grain (Zhang et al. 2012). Due to key role of VIT in Fe distribution of cereal grains, they can act as a potential target in genetic biofortification.

Structurally VIT transporter is a dimeric protein where each monomer consists of 5-TMD and a cytoplasmic MBD (Kato et al. 2019). The respective N-and C-termini of transporter protein are located towards cytoplasm and lumen of vacuole. The MBD of VIT transporter constitutes 3-helical bundles which allow capturing of cytoplasmic Fe^{2+} ions from chelating molecule. The ion translocating pathway of transporter protein is present at dimer interface with the presence of conserved methionine and carboxylate residues that facilitate efficient transport of Fe to vacuole. Further, transmembrane kinks due to the presence of proline and glycine residues on TMD1 and 2 are highly conserved among CCC1/VIT1 family transporters (Kato et al. 2019).

3.10 Natural Resistance-Associated Macrophages Protein (NRAMP) Family

NRAMP family is a diverse class of integral membrane proteins with members present in bacteria, fungi, animals and plants. They function in both inter- and intracellular trafficking of a wide range of divalent metal ions such as Fe^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Ca^{2+} and Pb^{2+} (Gunshin et al. 1997). Studies on mouse Nramp2 revealed structural similarity of NRAMP protein with Slc 11. NRAMP protein contains 10–12 TMD with a twofold inverted symmetry like LeuT superfamily (Frickey and Lupas 2004). Further, the conserved hydrophobic

core of 10TMD is divided into two direct repeats with TMD1–5 helix repeats have inverted topologies (Cellier 2012; Czachorowski et al. 2009). The presence of Nramp-specific polar residues within TMD1 and TMD6 has also been reported in phylogenetic studies (Gu and Vander Velden 2002; Courville et al. 2008). In higher plants, NRAMPs play an essential role in metal homeostasis. The first plant NRAMP member is identified in *Arabidopsis*, i.e. AtNRAMP1 function as high-affinity Mn^{2+} transporter, and plays an essential role in Fe homeostasis *in planta* (Curie et al. 2000; Cailliatte et al. 2010). Functional homologs of NRAMP genes have been identified in *Arabidopsis* (At NRAMP 1–6), rice (OsNRAMP 1–7), common bean (Pv NRAMP 1–7) on organellar membrane and cell membrane, which are responsible for translocation of divalent metal ions to various sinks (Thomine et al. 2000; Belouchi et al. 1997; Ishida et al. 2018). But some members such as Nrat1 and EIN2 are involved in Al^{3+} transport and ethylene signal transduction (Lu et al. 2018; Alonso et al. 1999).

3.11 OPT/YSL Family

Oligopeptide transporters (OPT) are novel family of transporters which are involved in transport of amino acids and oligopeptides (tri-penta peptides). In addition to peptide substrates, a subfamily of OPT, i.e. yellow stripe 1-like (YSL), mediates transport of metal complexes with peptides/amino acids across cellular membrane. Basically, YSL functions as proton-coupled symporter of metal-chelate complex. Their differential selectivity for metal substrates is dependent on extracellular loop between TMD6 and 7 (Harada et al. 2007).

The first member identified of YSL/OPT family is ZmYS1, mutation of which causes yellow stripes on maize leaves. These yellow stripes signify interveinal chlorosis which is resulting from defective Fe uptake, from which the family gets its name (Curie et al. 2001). Studies on ZmYS1 reveal function of this transporter in transport of Fe-phytosiderophore complexes from root cell exterior to cytoplasmic interior and further its symplastic loading to vasculature. Other metal cations such as Zn^{2+}, Cu^{2+} and Ni^{2+}, etc. are also transported by ZmYS1. Multiple YSL genes or their putative homologs have been identified in plants with 18 members in rice (Koike et al. 2004), 8 in *Arabidopsis* (DiDonato Jr et al. 2004), 5 in peanut (Xiong et al. 2013) and 67 in wheat (Kumar et al. 2019) which mediate transport Fe^{2+}-NA/Fe^{3+}-MAs or other metal chelates to various tissues and have a key role in metal homeostasis. Among them, AtOPT3, AtYSL1 and AtYSL3. OsYSL2, TcOPT3, etc. are known to be involved in phloem loading of Fe, Zn and other mimic metal cations in sink tissues (such as young leaves, developing fruits and seeds) for their accumulation (Zhai et al. 2014; Waters et al. 2006; Chu et al. 2010; Ishimaru et al. 2010; Hu et al. 2012). In addition to the above, other transporter families such as plant cadmium resistance, multidrug and toxic compound extrusion family, etc. have also been reported to regulate metal ion flux in plants.

3.12 Sequestration and Accumulation of Micronutrients in Vacuolar Compartments

The unique ionome composition of different cell types in each plant organ (i.e. root, shoot, leaf, flower, etc.) suggests that cell-specific accumulation and tissue distributions of metal nutrients are under strict regulation to maintain metal homeostasis. It is thought that such regulation contributes to distinct physiology of particular cell type. In this regard, the subcellular organelle vacuole is central for sequestration of metal cations, resulting in maintenance of their plasmatic concentration and further detoxification. This property of vacuolar compartments, to act as metal store house, is necessary for optimum cellular functioning. The compartmentalization of essential micronutrients/heavy metal cations in vacuoles depends upon the functioning of tonoplast transporters and vacuolar pumps (i.e. members of VIT, CAX and NRAMP family). Hyperaccumulators such as *Arabidopsis halleri*, *Thlaspi caerulescens*, *Dichapetalum gelonioides*, etc. are found to accumulate high concentrations of Zn^{2+}, Ni^{2+}, Cd^{2+}, etc. in root vacuoles just like non-hyperaccumulators, but they differ in metal accumulation by aerial shoots which is significantly higher in hyperaccumulator species due to increased expression of HMA proteins controlling long-distance vascular transport of micronutrient metals.

Sequestration of micronutrient metal ions in leaf vacuoles is one of the tolerance mechanisms to favour hyperaccumulation and detoxification in metal hyperaccumulators. This also maintains nutrient supply in desired sinks at the time of need. Despite of NA, MAs, histidine and organic acids (such as citrate, malate, etc.) form chelating complexes with heavy metal micronutrients in subcellular compartments including vacuole. In addition, cysteine-rich protein entities, phytochelatins (PCs) and metallothioneins (MTs) are best characterized for their metal-binding properties in plants. Despite of ubiquitous presence of MTs in animal and plants, they share a common feature of heavy metal homeostasis with PCs. MTs are gene-encoded low-molecular-weight peptides with a high percentage (20–30%) of cysteine residues responsible for metal binding. In contrast, PCs are enzymatically synthesized peptides having general formula γ-(glutamic acid-cysteine)$_n$ where $n = 2–11$ with great affinity for heavy metal ions (Shukla et al. 2016). The sulfhydryl group of cysteine moieties in PCs and MTs reacts with free metal ions in cell cytoplasm and form low-molecular-weight complexes. These PC-metal/MT-metal ion complexes are then transported to vacuolar compartments for their detoxification.

4 Journey of Micronutrients to Seed (Grain) Sinks: Long-Distance Phloem Transport of Micronutrients

The charged nature of micronutrient metal ions results in their specialized vascular transport from root to shoot and then from shoot to other vegetative/reproductive sinks. There is transition in a form of micronutrients absorbed, during the long-

distance transport from root xylem to leaf phloem of minor veins, which affects their mobility in phloem. In contrast to predominance of divalent cationic or metal-organic acid form of micronutrients in xylem sap (pH ≈ 5.5), they are generally present in bound chelated form as metal NA, metal-DMA/phytosiderophores in phloem sap (pH ≈ 7.3–8.5) to avoid their precipitation during delivery to sink tissues. It is well known that phloem is the main translocating tissue which supplies sugars and mineral nutrients to developing plant sinks irrespective of their location. However, the loading of micronutrients in phloem at site of source (i.e. leaf mesophyll/xylem vessel at minor veins) can be apoplastic or symplastic depending upon the activity of associated companion cell. There can be a direct loading of metal cations from xylem into phloem parenchyma in minor veins due to their close proximity. Transfer cells in phloem mediate such apoplastic loading of micronutrients. The invaginated wall growths and numerous membrane transporters on transfer cells favour greater nutrient fluxes (Sondergaard et al. 2004). The presence of membrane H^+-ATPase further boosts up secondary active transport of nutrients in apoplastic loaders. Another route is the symplastic loading of metal chelates (metal NA, metal-DMA/phytosiderophores, metal PCs) from mesophyll cells into intermediary cells of sieve element complex via plasmodesmatal connections.

Mutation studies and researches on metal tolerance mechanism of hyperaccumulators revealed that nonproteinogenic amino acid NA is most favoured organic ligand for several micronutrient metals in phloem. NA is also found to form stable complexes with Mn^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+} in vitro (Anderegg and Ripperger 1989). Overexpression of NA biosynthetic genes, i.e. nicotianamine synthase (NAS) in soybean (Nozoye et al. 2014), sweet potato (Nozoye et al. 2017), tobacco (Kim et al. 2005), rice (Masuda et al. 2009; Lee et al. 2011), etc. through transgenics, has been found to increase the Fe and Zn concentration of leaves and respective sink organs (i.e. root in sweet potato and seeds in rice and soybean). These investigations also support the role of NA in shoot to root signalling of iron and its remobilization from mature to developing tissues. But NA is the sole player in metal translocation; this assumption has been negated with discovery of OsYSL15 in rice which performs dual function of phloem translocation of Fe^{3+}-DMA in addition to its rhizospheric uptake (Inoue et al. 2009). So other metal ligands such as DMA, histidine and phytochelatins have also been associated in phloem translocation of micronutrients.

In plants, the immature organs act as sinks during their early growth and depend upon source for organic and inorganic nutrition. During a particular growth stage, there can be more than one sink for the source leaves, so the allocation/partitioning of nutrients to diverse sinks decides their accumulation in desired sinks under such situation. Thus, in food crops where grains or seeds are economic sinks, total nutrient concentrations in phloem and its distribution towards developing grains are of equal importance. But if the whole shoots are to be consumed as human or animal food, then the total micronutrient contents of shoot matter the most than nutrient allocation. Nutrient remobilization from mature leaves to developing sinks during senescence also boosts the supply of micronutrients. The role of some senescence-

responsive genes in micronutrient loading of seeds has also demonstrated. One such example is NAM genes in wheat whose reduced expression was found to decline nutrient partitioning to grain and lowered grain Fe and Zn contents (Waters et al. 2009).

Another key molecular player in micronutrient transport to the sinks (grains/leaves) is YSL transporter family. As mentioned earlier, YSL transporters are involved in transport of metal chelates especially NA-metal complex. YSLs of *Arabidopsis*, particularly AtYSL1 and AtYSL3, are involved in delivery of Fe, Zn, Cu and Mn to reproductive organs. Mutations in these YSL genes cause impaired vascular transport of metal micronutrients during senescence and thus limit metal accumulation in seed. Similarly, YSL ortholog in rice OsYSL2 mediates phloem transport of Fe and Mn in aerial shoots and metal loading in seeds (Ishimaru et al. 2010). Thus, better understanding on regulation of such metal transporters in long-distance micronutrient signalling will help in bioengineering of staple crops with high micronutrient density.

5 Future Scenario: The Way Ahead

Although recent tools of molecular breeding are continuously harnessing genetic diversity of wild germplasm to enhance micronutrient density of food crops, there is a need to find cell-specific and developmental-stage-specific regulators controlling the metal-oriented circuits to maintain homeostasis. The complex interactions of these essential micronutrients with toxic mimic cations (such as Pb, Cd, Hg, etc.) and other macronutrients (N, P, S, etc.) need to be explored more in order to enhance metal absorption in edible sinks. Inclusion of modern system biology and omics approaches in biofortification studies will be able to enhance our understanding on mechanism of root to shoot signalling with better regulation of metal loading in seed sinks. Understanding transcriptional and post-transcriptional regulation of genes encoding transporter proteins can be another promising research avenue which will help us to increase uptake and translocation micronutrients in seed. In addition to the above, novel senescence-associated genes should be identified to facilitate metal remobilization towards developing sinks.

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