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Nutritional Quality Improvement in Plants

Springer

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Preface

Plant-based foods provide macronutrients (carbohydrates, proteins and lipids), micronutrients (minerals and vitamins) and phytonutrients (isoprenoids, polyphenols, etc.) for growth, development and well-being of humans and animals. Two-thirds of the world population, especially in the developing countries, are dependent on staple food crops (rice, wheat, maize, cassava and potato) which are calorie-rich and hyper-glycemic but poor in micronutrients and other health-promoting substances. Deficiencies in micro- and phytonutrients are a global concern that cause stunting and underweightness among children (under the age of five), permanent physical and mental impairment, affect work performance and increase the risk of mortality by infectious and chronic diseases like cardiovascular diseases, type 2 diabetes and certain types of cancers. Rise in population and global climate change have further exaggerated the problem, how to produce safe nutritious food in adequate amount with low inputs without affecting the environment? The novel tools and techniques of agronomy, conventional and molecular breeding (marker-assisted selection, association genetics, QTL), metabolic engineering, -omics resources, RNAi [through small RNAs, short interfering RNA (siRNA); microRNA (miRNA) and artificial microRNA (amiRNA)] or antisense and genome editing (CRISPR/Cas) technologies have played a significant role in better understanding of the synthesis, uptake, transport and metabolism of macro- and micronutrients. This has led to the identification, isolation, characterization and cloning of novel genes for accumulation of nutrients (biofortification) and/or avoiding accumulation of undesirable substances in edible parts of the plant in sustainable and cost-effective manner without affecting yield, and farmer and consumer preference traits. During the last two decades, several food crops rich in some macro- and micronutrients, and phytonutrients have been developed by both conventional and molecular breeding, and metabolic engineering through transgenesis to improve the health and protect the poor rural populations in developing countries from chronic diseases. As per the HarvestPlus estimates, more than 20 million people are now growing and consuming biofortified crops developed through breeding. The present book contains chapters on the biofortification of crop plants (especially rice, maize, millets, brassica and potato) with essential

micronutrients (Fe, Zn, I, Ca, essential amino acids, provitamin A, vitamin B (B1, B_6 and B_9), C and E) and avoiding antinutrients (phytate, acrylamide and aflatoxins) through conventional and metabolic engineering tools.

Humans and animals are dependent on plants for all the essential minerals required for their optimal health and productivity. Plants do not synthesize minerals but acquire them from soil through a complex mechanism of their uptake, transport and accumulation in edible parts. Agronomic interventions (application of biostimulants and fertilizers to soil or foliar spray), conventional breeding and genetic engineering have the potential to enhance mineral content in plants. Conventional breeding has developed iron (Fe)-rich bean and pearl millet varieties which have improved iron status in women in Rwanda and of school children in India. Though plant breeding requires a long gestation period, it has a higher acceptance by farmers and consumers to provide minerals in more suitable and cost-effective ways with easy regulation. However, enhancing iron in polished rice at target levels is feasible only by genetic engineering in a rapid and cost-effective manner but with limited public acceptance and restricted regulations. **Tsakirpaloglou et al.** summarize the Fe biofortification by transgenic approach and also zinc (Zn) biofortification in rice polished grains through conventional and transgenic breeding efforts. They also discussed the contribution of Zn-rich rice in eradicating its deficiency by examining the bioavailability aspects as well as the retention of Zn content in rice grains after cooking. **Yadav et al.** summarize the impact of microbiota in iron acquisition by plants in addition to the conventional and transgenic efforts towards phytobiofortification for alleviation of iron deficiency in the global populace.

Calcium (Ca) is an essential macronutrient for plants and animals with key structural and signalling roles. Millions of people in developing and developed countries suffer from low dietary intake of Ca that leads to rickets and osteoporosis diseases. Most of the staple food crops are poor in calcium but finger millet (*Eleusine coracana* (L.) Gaertn.), an orphan crop contains exceptionally high calcium (376–515mg/100g grains) and thus offers prospects for biofortification breeding. The molecular mechanisms underlying the uptake, transport and accumulation of calcium in grains and existing genetic variation play an importance role for development of calcium biofortified crops. **Sharma et al.** discuss the role of high-throughput genotyping technology and phenotyping platforms in unravelling the genetic basis of complex traits such as calcium nutrition in finger millet.

Iodine, a non-metal micronutrient, is essential for human health and well-being. Human and animals obtain iodine mostly from diet and require a recommended daily allowance of 150 μg. Nevertheless, its deficiency is prevalent worldwide and is the cause of goitre, foetal damage, prenatal and infant mortality, irreversible mental retardation and brain damage. **Davila-Rangel et al.** highlight the different techniques and results obtained in developing crop plants biofortified with iodine.

Essential amino acids and micronutrients that are essential for normal growth and metabolism are not synthesised by the human body and must be obtained from diet. Inefficient intake of essential amino acids (protein energy malnutrition) causes child stunting and affects brain function and immune system. Maize, the third most

important cereal crop, is used extensively as human food and livestock feed across the world. Traditional maize possess poor endosperm protein with low levels of essential amino acids, lysine and tryptophan and micronutrients especially provitamin A (proA). **F. Hossain and coworkers** present an overview of the conventional, marker-assisted back-cross breeding and transgenic approaches in enhancing the quality of protein as well as the proA content in maize. Further, double biofortification with these two diverse micronutrients by either sequential or simultaneous marker-assisted stacking of genes, *o2* (for quality protein maize) and two genes, *lycopene ε-cyclase* (*lcyE*) and *β-carotene hydroxylase1* (*crtRB1*) (for proA) increased both essential amino acids, lysine and tryptophan more than twofold and proA concentration by 4.5-fold in the same cultivar. The stability of provitamin A in quality protein maize, their impact in reducing protein and vitamin A deficiencies, scope and challenges in dissemination have also been discussed.

Racio Diaz dela Garga et al. summarize the current knowledge on **folate** metabolism (biosynthesis in different subcellular localizations, and its degradation and stability) and regulation for its metabolic engineering to enhance its contents in model (arabidopsis) and crop (tomato, rice, potato, common bean, maize and lettuce) plants. The bioavailability of folate in biofortified crops and their socioeconomics is also presented.

Thiamine (vitamin B_1) in its active form, thiamine pyrophosphate (TPP), functions as an essential cofactor of key enzymes of the central metabolism. The plants are the main source of thiamine for humans. Most of the staple food crops contain thiamine in very low amounts that results in its deficiency in humans which causes a chronic disease called beriberi. **Yusof** highlights the role of thiamine in plant growth and stress tolerance as well as current progress in its biosynthesis and regulation to enhance its content in plants. However, many aspects of thiamine metabolism are still not fully understood, resulting in slow progress in improving its content in plants especially staple food crops.

Sainger and coworkers summarize the role, metabolism, and logic for biofortification along with current advances in breeding and metabolic engineering to improve the contents of three relevant vitamins, B_6, C and E in plants. These vitamins' biosynthesis and their regulation are well outlined to enhance them in sufficient amounts without affecting plant yield and preference traits for those in need. Their enhancements confer stress resistance and improve nutritional quality of plants for human health benefits.

Plants produce a variety of small organic molecules, the secondary metabolites that are not directly involved in basic metabolic processes but are responsible for taste, flavour, smell, colour or the protection of plants against herbivores (antifeedant) and microbial infections (phytoalexin) and abiotic stresses. These phytochemicals have important significance as an attractant (pigments or scents) for pollinators and seed-dispersing animals, allelopathic agents, food (carotenoids, flavonoids, phenolics) and pharmaceutical (anticancer agents, antimalarial compounds, etc.) for human health. **Garcia-Mier et al.** review the relevant and current literature on the use of various techniques like metabolic engineering,

nanostructures and/or nanomaterials, biostimulators, biocontrollers and elicitation on the production of secondary metabolites for agronomic and human health interest.

Brassica crops are good sources of oil, protein-rich seed meal and vegetables. Their seeds accumulate sulphur-rich secondary compounds called glucosinolates that protect plants from biotic and abiotic stresses. High glucosinolates in seed meal are bitter and unpalatable to poultry and livestock. Brassica vegetables in human diet are good source of highly beneficial glucosinolates (glucoraphanin). **Bist and Augustine** describe the basics of glucosinolates, their biological effects in addition to the efforts and strategies to reduce antinutritional glucosinolates and enhance desirable glucosinolate (glucoraphanin) content in brassica crops to improve their food and feed values.

Aflatoxins are toxic and carcinogenic secondary metabolites produced by certain *Aspergillus* species, *A. flavus* and *A. parasticus*, on infection of various important food and feed crops, especially maize, cotton, groundnut, tree nuts, etc. Consumption of aflatoxin-contaminated food grains and feeds not only cause serious health problems in humans and livestock but also reduce food and feed values leading to significant economic losses worldwide. **Pooja and coworkers** provide comprehensive overview on the various strategies and advances in aflatoxin resistance in crop plants. The various factors affecting aflatoxins contamination, its control cultural practices and biological agents, identification of molecular markers and QTLs associated with aflatoxin resistance and genetic engineering through overexpression and host-induced gene silencing of aflatoxin biosynthesis genes are discussed to develop durable aflatoxin resistant in crop varieties.

Acrylamide, a suspected carcinogen and neurotoxin, is formed from free asparagine and reducing sugars in Maillard reaction during high-temperature cooking and processing of potato (French fries, chips, etc.), baked cereal products (bread, biscuits, etc.), coffee and chocolate. Consumption of these food products results in dietary intake of 0.3–0.7 μg acrylamide $kg^{-1}day^{-1}$. The acrylamide-forming potential depends on free asparagine content which is affected by genotype (G), environmental conditions (E) and their interaction (G x E). Significant differences that exist in free asparagine and sugar concentrations between varieties in all crops have helped in identifying the molecular markers/QTLs associated with them to expedite their breeding. Further agronomic (ensuring adequate sulphur fertilization in relation to nitrogen supply) and genetic engineering approaches being used to reduce the free asparagine and sugar concentration are discussed by **Raffan and Halford** in their chapter. Reducing acrylamide-forming potential of crops enables food industries to comply with the regulatory system.

Phosphorus is stored in the seeds in the form of phytic acid and its salt (phytate). Phytate is considered antinutrient as it binds to important mineral nutrients like iron, zinc, calcium and magnesium that are not hydrolysed and absorbed in humans and monogastric animal's gut due to the absence of the digestive enzyme, phytase, and are thus excreted to the environment causing the loss of minerals from humans and animals and pollution of waterways. To solve these problems, the total phosphorus accumulation and phytate concentration in grains is to be reduced.

Generation of low phytate mutants and genetic engineering (RNAi or CRISPR/Cas) and use of phytases have reduced phytate level and thus offer potential to grow crops with low phosphorus fertilizers, increase bioavailability of mineral nutrients for human and animal health, and reduce water pollution. **Kaul and coworkers** discuss the production of low phytic acid crops using various approaches especially the use of phytases. Phytate being an antioxidant, anticancer and anticalcification agent has cautioned decrease in its level in crop plants.

Pearl millet is an ideal biofortification staple food crop for more than 90 million poor farmers in arid and semi-arid regions of West and East Africa and India. This crop is highly productive under harsh conditions including infertile soil with high pH, high Al^{3+} saturation and low moisture content, high temperature, high salinity and restricted rainfall. This highly nutritious cereal with large naturally occurring genetic variability for micronutrients (Fe and Zn) has been exploited by the International Crops Research Institute for Semi-Arid Tropics (ICRISAT) with HarvestPlus support for the improvement of grain iron with high yield or farmer-preferred agronomic traits. **Vinoth and Ravindhran** describe the development of high-iron pearl millet through conventional breeding and also discuss their efficacy, consumer acceptance and cost-effectiveness.

Potato is a global, low-cost vegetatively propagated non-grain food crop that supplies starch, protein, vitamins and minerals in human diet and can play a pivotal role in addressing malnutrition problem. **Som Dutt and coworkers** describe the improvement in its nutritional quality and the processing attributes (processed tuber texture, cold-induced sweetening, browning of sliced tubers) using the biotechnological approaches. Improvement achieved in a number of nutrients, (e.g. starch, protein, essential amino acids, provitamin A, vitamin C and E, minerals and phytonutrient contents) and decrease in the antinutrients (e.g. glycoalkaloids, acrylamide and other allergens) through metabolic engineering are highlighted. The role of gene editing, CRISPR/Cas, in improving potato nutritional qualities is also discussed.

In most of the above cases, efforts have been made to increase a particular micronutrient though most of our staple food crops are deficient in several of them. This signifies for an urgent need of multiple biofortification for simultaneous enrichment of many nutrients to produce nutritionally complete crops by stacking of corresponding nutrient metabolic/regulatory genes either by plant breeding or metabolic engineering or both, including genome editing technologies without negative impact on their yield and environment. Simultaneously, the improved nutritional traits should also improve agronomic or producer traits for widespread adoption by farmers. Beside this, nutrient stability, nutrient bioavailability and absorption as well as cooking and sensory quality of biofortified crops along with their social and economic aspects (cost-effectiveness and acceptance) and ethical issues should also be explored. There is a need to move these crops from proof-of-principle to products through government/private investment to ensure sustained health benefits to consumers. Bioinformatics deals with the tools and techniques of capturing, managing, analysing and integrating the huge amounts of genomics, transcriptomics, proteomics and metabolomics data for the better

understanding of the processes and mechanisms to improve nutritional value and yield of plants. **M. Dangi and co-workers** discuss the role and applications of bioinformatics in gene network analysis and crop improvement.

The book chapters are written by the experts in the field and provide intuitive accounts on the various aspects of nutritional enhancement of plants. The book is a valuable resource for scientists, researchers, students, planners and industrialists working in the area of agriculture, plant sciences, agronomy, horticulture, plant physiology, molecular biology, biotechnology, food and nutrition, soil and environmental sciences. We are indebted to the contributors for their efforts in preparing intuitive accounts of various aspects of knowledge in this area. We express our sincere thanks and gratitude to all these colleagues and warm appreciation and thanks to Springer Nature publisher for their keen interest in bringing out this title with quality work. We are also thankful to our family members and Ph.D. students for their understanding and patience during planning and preparation of this title.

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Contents

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Chapter 1
Biofortified Zn and Fe Rice: Potential Contribution for Dietary Mineral and Human Health

Nikolaos Tsakirpaloglou, B. P. Mallikarjuna Swamy, Cecilia Acuin and Inez H. Slamet-Loedin

Abstract Iron (Fe) and zinc (Zn) deficiency constitute a major micronutrient deficiency around the globe, affecting rural populations residing in developing countries with minimum purchasing power and/or access to a diverse diet. Biofortification, the enrichment of staple food/crops with bioavailable micronutrients or vitamins in their edible parts, provides a potential sustainable solution towards such issues, in combination with other existing efforts. Utilisation of rice as a platform for the delivery of products biofortified with Fe and Zn could impact greatly the livelihood of people dependent on rice-based agri-food systems globally. The HarvestPlus and its partners have successfully supported the production, deployment and release of conventionally bred Zn-biofortified lines of rice and wheat in several countries; and also support potential innovative approaches such as genetic engineering and genome editing with higher Fe and Zn content in the grain. A large number of reviews on iron biofortification in rice has been published, in this review we summarise the Fe biofortification by transgenic approaches, but the major focus of this review is on the conventional and transgenic breeding efforts to generate Zn-biofortified lines in rice and discuss their potential to contribute in eradicating Zn deficiency, by examining bioavailability aspects, as well as the retention of Zn content in rice grains after cooking. We additionally examine the importance of a clear pathway for the successful delivery and large-scale adoption of high-Zn rice to achieve maximum impact.

Keywords Biofortification · Rice · Fe · Zn · Malnutrition · Breeding · GMOs · DALYs

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1.1 Introduction

Globally, Fe and Zn deficiency is widespread particularly in rural and developing regions where people consume cereal-based diets and have less opportunities for diet diversification; however, its presence is also detected in prosperous areas where diets are unbalanced, contributing to 'hidden hunger' (Kennedy et al. 2003; Muthayya et al. 2013; Roohani et al. 2013). It is estimated that micronutrient deficiencies affect approximately 1.6 billion people globally, particularly children, pregnant and lactating women, and in low- and middle-income countries can cause economic losses ranging from 2 to 5% of gross domestic product (GDP) (Black 2003; Darnton-Hill et al. 2017). A study showed that the inadequate Zn intake was positively correlated with the prevalence of stunting and that in countries identified as being at low, moderate and high risk of inadequate zinc intake its prevalence was 19.6, 28.8 and 43.2%, respectively (Wessells and Brown 2012).

Zn deficiency is a major cause of stunting in children, largely because Zn plays a major part in cell division, cell growth, wound healing, and the breakdown of carbohydrates, as well as the regulation of the innate and adaptive immune system (Sanna et al. 2018). The symptoms for Zn depletion may vary with age from diarrhoea in early infancy to stunted growth and childhood morbidity and mortality at a later stage of development. Moreover, it can lead to weakened cognitive function, behavioural issues, impaired memory, learning disability and neural atrophy (International Zinc Nutrition Consultative Group (IZiNCG) 2004). Additionally, Zn has been found to protect the prostate gland from prostatitis and prostatic hypertrophy in males (Cui et al. 2015); it affects sperm count and mobility as well as levels of serum testosterone (Zhao et al. 2016).

Iron is an important micronutrient in plants and required in various physiological processes including respiration and photosynthesis. Different strategies are known for the uptake of iron from the rhizosphere in higher plants. (a) Strategy I (non-Graminaceae) involving ferric chelate reduction and absorption of ferrous irons at the root surface and plasma membrane, respectively, and (b) Strategy II (Graminaceae) includes mugineic acid (MA) biosynthesis and secretion or (c) a combination of both (Connorton et al. 2017). The initial attempt for iron biofortification (Goto et al. 1999) started long earlier than zinc biofortification efforts even though only recently reached the nutritional target under field condition (Trijatmiko et al. 2016). The mechanisms and the pathway of Fe uptake and translocation in rice have been extensively reviewed (Bashir et al. 2010, 2013; Kobayashi and Nishizawa 2012, Slamet-Loedin et al. 2015). Here, we summarise most of the transgenic studies to develop Fe-biofortified rice and its respective inserted genes (Ludwig and Slamet-Loedin 2019) in Table 1.1.

Zinc (Zn) is a metallo-mineral essential for the growth, development and survival of plants and animals (King and Cousins 2006). It is localised ubiquitously within the cells and acts as a major co-factor for more than 300 enzymes involved in catalytic and regulatory biochemical reactions in the human body (King and Cousins 2006). It plays an important role in the structural and functional integrity of many proteins, and thereby regulates vital biological reactions in the cells such as DNA

Table 1.1 Transgenic Fe-biofortified rice

Gene	Iron ([c] in ppm) polished/brown		Growth condition	References
	TG	WT		
OsIRT1	~12	~10	Paddy field	Lee and An (2009)
TOM1	~18	~15	Hydroponic	Nozoye et al. (2011)
OsYSL15	~14	~12	Paddy field	Lee et al. (2009a)
OsNAS1	up to ~19	~12	Field	Zheng et al. (2010)
OsNAS2	~10	~4	Greenhouse	Lee et al. (2011)
OsNAS3	~12	~4	Greenhouse	Lee et al. (2009b)
OsYSL2	~7.5	~1.8	Glasshouse	Ishimaru et al. (2010)
OsNAS1, OsNAS2, OSNAS3	up to ~19	~4.5	Glasshouse	Johnson et al. (2011)
HvNAS1	~8.5	~4	Greenhouse	Masuda et al. (2009)
SoyferH1	up to ~25	~17	Greenhouse	Qu et al. (2005)
SoyferH1	~18	~18	Greenhouse	Drakakaki et al. (2000)
SoyferH1	up to ~37	~10	Screenhouse	Vasconcelos et al. (2003)
SoyferH1	up to ~16	~6.75	Greenhouse	Paul et al. (2014)
SoyferH1	up to ~9.2	~3.8	Greenhouse	Khalekuzzaman et al. (2006)
SoyferH1	up to ~7.6	~3.3	Greenhouse	Oliva et al. (2014)
OsIRO2	up to ~15.5	~6	Greenhouse	Ogo et al. (2011)
OsVIT1	~26	~20	Paddy field	Zhang et al. (2012)
OsVIT2	~28	~20	Paddy field	Zhang et al. (2012)
PyFerritin, rgMT, phyA	~22	~10	Greenhouse	Lucca et al. (2002)
OsYSL2, SoyFerH2, HvNAS1	up to ~4	~1	Paddy field	Masuda et al. (2012)
HvNAS1, HvNAS1, HvNAAT, IDS3	up to ~7.3	~5.8	Paddy field	Suzuki et al. (2008)
HvNAS1, OsYSL2, SoyFerH2	~6.3 (~5.02)	~3.2 (~1.46)	Greenhouse	Aung et al. (2013)
AtNAS1, Pvferritin, Afphytase	up to ~7	~1	Hydrophonic	Wirth et al. (2009)
AtIRT1, PvFERRITIN, AtNAS1	up to ~10.46	~2.7	Greenhouse	Boonyaves et al. (2017)

(continued)

Table 1.1 (continued)

Gene	Iron ([c] in ppm) polished/brown		Growth condition	References
	TG	WT		
GmFERRITIN, OsNAS2	~15	~2.5	Field	Trijatmiko et al. (2016)
AtNAS1, AtFRD3, PvFer	up to ~11.08	~2.05	Greenhouse	Wu et al. (2018)
AtNAS1, PvFer, AtNRAMP3	up to ~13.65	~2.72	Greenhouse	Wu et al. (2019)
OsNAS1, HvHAATb	~up to 18	~4	Hydroponic	Banakar et al. (2017)

and RNA synthesis, activation of transcription factors, RNA polymerases and reverse transcriptase (Cousins et al. 2006; Bashir et al. 2013; Olsen and Palmgren 2014). Zn is essential in human health and nutrition, with approximately 3000 Zn-binding proteins found in the human body (Andreini et al. 2006).

Unlike Fe, Zn does not have a specialised storage system in the body, so sufficient intake of Zn is required daily to maintain a healthy life. Strategies to reduce Zn deficiency include supplementation as well as food-based approaches such as diet diversification, food fortification and biofortification. Addressing micronutrient malnutrition, improve maternal health and reduce child mortalities are important to achieve sustainable development goals globally by 2035 (United Nations 2018).

Biofortification of cereals including rice, wheat and maize has been considered one of the most economical, and sustainable among the food-based approaches to tackle malnutrition. It can benefit all sections of the populations, especially the urban and rural poor, who may find it difficult to access other health and nutrition interventions (Bouis et al. 2011). Zn in rice is retained in significant quantities even after polishing and cooking and can be made easily available for human consumption (Oghbaei and Prakash 2016). However, most of the modern rice varieties have suboptimal levels of Zn in the endosperm and therefore do not provide sufficient Zn for the recommended daily dietary intake (Bouis et al. 2011). Fortunately, studies have shown that existing variations for Zn micronutrients in the grain within the rice germplasm potentially provide sufficient source for breeding programmes to increase Zn levels in rice (Swamy et al. 2016). As part of the HarvestPlus programme, these high-Zn rice varieties, containing increased Zn in polished grains compared to ordinary varieties, have been commercialised in Bangladesh, India and the Philippines. However, achieving the desired level to have a significant contribution to the recommended levels of daily intake of Zn in staples has been slow due to complex genetic basis and huge environmental effects (Mahender et al. 2016).

Using genetic engineering approaches, target levels of Zn have been increased up to 4–5-folds from the basal level (Johnson et al. 2011; Trijatmiko et al. 2016), but its commercial release needs wider public acceptance and regulatory approvals in the target countries (Trijatmiko et al. 2016). Thus, both conventional and transgenic

high-Zn rice can be a potential source of dietary Zn, which could improve the health and nutrition of malnourished populations in Asia or elsewhere.

The challenge of biofortification among other factors is not only to secure the increased levels for per capita intake of Zn but to sufficiently shift the Zn status prevalence in the target community. This will depend on the nutrient availability, efficacy as well as wider adoption and consumption of the high-Zn rice varieties. A holistic Zn breeding approach both by conventional and modern technologies, starting from trait development, and followed by product development and dissemination, is essential to achieve a wider impact on health and nutrition. Here, we discuss the potential of rice biofortification as a sustainable source of dietary Zn, we present the breeding efforts through conventional and genetic modification approaches to enhance Zn levels in polished grains, and we examine the various factors affecting the Zn bioavailability and bioefficacy.

1.2 Global Pattern of Zinc Deficiency and the Role of Biofortification in Human Nutrition

1.2.1 Rice Consumption and Zinc Deficiency

Rice feeds more than fifty per cent of the world's population. An estimated 475 million metric tons of rice were consumed globally during 2016/2017 and predicted to increase further (rice consumption worldwide in 2016/2017). Rice provides approximately 19 and 13% of global human per capita energy and per capita protein, respectively (IRRI World rice statistics online). The global use of rice exceeds 50 kg per capita, but in many Asian countries such as Bangladesh, Indonesia, Cambodia, Myanmar, Vietnam and the Philippines, the per capita rice consumption of rice is more than 100 kg annually. Although the consumption of rice outside Asia is lower, it continues to grow rapidly in many countries of Africa and Latin America (Muthayya et al. 2014). Because of such high consumption, rice constitutes an ideal vehicle for delivering micronutrients, including Zn, at a large scale.

However, rice has a very low concentration of Zn with the baseline amounting to 12–16 $\mu g\ g^{-1}$ in polished grains (Bouis et al. 2011). In addition, cereals are rich in phytate which reduces the absorption of Zn in human body (Prasad 2008). In developing nations, at least 60% of the dietary Zn is derived from major cereals and legumes (Liu et al. 2017). Hence, people dependent on major cereals such as rice, wheat and maize for their daily caloric intake and nutritional needs often suffer from Zn deficiency (Pingali 2012; Fanzo 2015). The highest risk for inadequate zinc intake has been identified in countries in South and Southeast Asia, Sub-Saharan Africa and Central America due to lower zinc availability in their food supplies, the limited intake of animal source foods and the high content of phytate in the diet. Inadequate dietary zinc intake in Sub-Saharan Africa and South Asia constitutes

clearly a public health concern and positively correlated with stunting in children (Wessells and Brown 2012).

Polished rice whether it is consumed as white rice or parboiled is often the preferred staple in countries with medium to the high prevalence of Zn deficiencies (Dipti et al. 2012). Hence, biofortification of rice with zinc is a key strategy to address Zn deficiency malnutrition.

1.2.2 Biofortification of Staple Crops to Improve Human Nutrition

Biofortification is the concept of delivering micronutrients via staple foods through agronomic practices, conventional plant breeding or modern biotechnology. It has been recognised 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 2009). Biofortification provides an alternative to reach subgroups of the population where supplementation and conventional fortification activities difficult to implement. These population groups often have limited purchasing power to access a nutrient-rich diverse diet (Hefferon 2015; Singh et al. 2016).

Over the past decade, a number of biofortified crops (including rice, beans, sweet potato, cassava and legumes) have been developed through the HarvestPlus biofortification programme, utilising conventional breeding approaches. For Zn biofortification, wheat and rice are the major target crops (Bouis and Saltzman 2017) (Fig. 1.1).

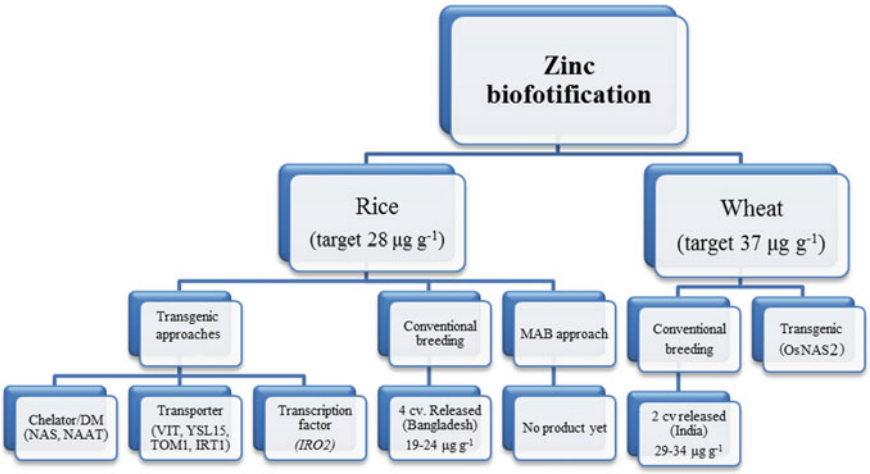

Fig. 1.1 Zinc biofortification of staple food crops, including rice and wheat, has been a major activity for the HarvestPlus programme (www.harvestplus.org)

Recently, the HarvestPlus updated their nutritional target for Zn in polished rice to 28 $\mu g\ g^{-1}$ in milled rice (Bouis and Saltzman 2017) or an additional of 12 $\mu g\ g^{-1}$ of Zn into commercial rice varieties from baselines of around 14–16 $\mu g\ g^{-1}$. This calculation is based on reaching approximately 40% of the Estimated Average Requirement (EAR) for non-pregnant, non-lactating women and children (4–6 years of age) and taking into account the loss of Zn during milling, cooking and bioavailability (Bouis et al. 2011; Bouis and Saltzman 2017).

The prospect of rice as a vehicle for biofortification of Zn can be inferred from a recent study conducted by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) which showed that consumption of micronutrient enriched rice significantly reduces Zn deficiency by up to 6% among ultra-poor women. The consumption of fortified rice also contributed to the reduction in morbidity among woman in Vulnerable Group Development (VGD) demonstrating the considerable impact of micronutrient improvement in rice (World Food Programme (WFP)). Thus, having biofortified rice grown locally in an adaptive cultivar is an attractive option to alleviate micronutrient deficiency.

1.3 Biofortification Approaches to Increase the Zinc Content in Rice Grains

1.3.1 Breeding Approaches

HarvestPlus in collaboration with IRRI, the International Centre for Tropical Agriculture (CIAT) and National Agricultural Research and Extension Systems (NARES) partners are implementing programmes to improve Zn content in rice varieties targeted to South Asia, South East Asia and Latin American countries (Bouis et al. 2014).

Rice has a large germplasm collection and its characterisation for Zn indicated the prevalence of wide genetic variation for grain Zn both in brown and milled rice, thus providing an opportunity to exploit this variation and subsequently breed for high-Zn rice varieties (Gregorio 2002; Neelamraju et al. 2012; Agarwal et al. 2014). A breeding target for the polished grain Zn has been set to 28 $\mu g\ g^{-1}$, whilst target countries beyond Bangladesh, India, Indonesia and the Philippines have been expanded to other South-East Asian countries such as Myanmar, Cambodia and Vietnam where both rice consumption and Zn deficiency levels are high. Currently, the high-Zn breeding programmes targeted to Asia are successful in developing moderately high-Zn rice varieties with a number of Zn-biofortified rice varieties that have been released in the Philippines, India and Bangladesh.

The major emphasis for conventional and/or molecular breeding approaches are the identification of new donors with high levels of Zn and acceptable yield potential, as well as the identification of major effect quantitative trait loci (QTLs) for grain Zn and development of high-Zn breeding lines with desirable grain quality traits

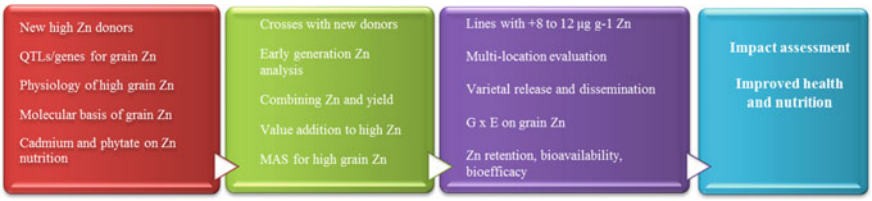

Fig. 1.2 Pathway for development and dissemination of high-zinc rice varieties

(Fig. 1.2). In our high-Zn breeding programme, high grain Zn rice germplasm from diverse sources having with more than 28 $\mu g\ g^{-1}$ of Zn, acceptable yield potential and consistent performance over seasons and geographic locations were identified. These new high-Zn donor lines are being extensively used in breeding programmes at IRRI and its national partners (Swamy et al. 2016). Moreover, the development of markers for grain Zn will enable more precise and quicker identification of high-Zn rice varieties through marker-assisted breeding (MAB). Significant efforts have been made over the last few years to understand the molecular basis of grain Zn accumulation in rice (Stangoulis et al. 2007; Chen et al. 2008; Naveen et al. 2014; Swamy et al. 2016). Several rice association panels, bi-parental and multi-parent mapping populations were evaluated for Zn content in the grain, and some major effect QTLs with high phenotypic variance and additive effects for grain Zn were identified on different chromosomes (Stangoulis et al. 2007; Naveen et al. 2014; Swamy et al. 2016). However, further QTL validation and diagnostic maker development are necessary for their effective use in MAB. Multi-parent populations and wild species derived advanced backcross populations have shown promising results for grain Zn and could be exploited in a more systematic way and in a large scale for the improvement of mineral nutrients in rice (Swamy et al. 2014; Descalsota et al. 2018).

Genome-wide expression analysis and targeted metal homoeostasis genes expression analysis on high-Zn breeding and parental lines are being carried out at IRRI. A set of genes, *OsMTP6*, *OsNAS3*, *OsMT2D*, *OsVIT1* and *OsNRAMP7* (Descalsota et al. 2018) were identified to be co-located with Fe and Zn QTLs, whilst markers surrounding OsNAS3 gene were suggested for application in the breeding programmes (Descalsota et al. 2018). Bayesian analysis showed that agronomic and yield component traits had no significant direct effect on grain Fe and Zn, so yield and Zn can be combined to develop high-Zn rice varieties. Gene expression analysis revealed up-regulation of *OsNAS1*, *OsNAS3* and *OsNAS2* in high-Zn donor lines in parallel, application of mutation breeding strategies and screening under Zn-deficient soil conditions is utilised to identify high-Zn mutant lines. Additionally, the exploitation of heterosis for grain Zn in hybrid rice breeding programmes holds great promise. Overall, our multi-pronged approach enables us to understand the genetic and molecular mechanisms involved in grain Zn accumulation and accelerate the development of high-Zn rice varieties. The high-Zn rice breeding programmes in some of the Asian countries have been successful in developing and releasing moderately high-

Zn rice varieties with an additional Zn of 6–8 μg g^{-1} (to the common baseline of 12–14 μg g^{-1} in popular varieties). The first generation of moderately high-Zn rice varieties released is BRRI dhan 62, BRRI dhan 64, BRRI dhan 72, BRRI dhan 74 and BRRI dhan 76 in Bangladesh, and NSICRc460 (Zinc Rice 1) in the Philippines, and DRR Dhan-45 and Chhattisgarh Zinc Rice 1 in India (Table 1.2). These varieties are found to exhibit good agronomic traits, yield potential, grain quality traits and resistance to insects/diseases (Table 1.3). Simultaneously, efforts to develop second and third generations of high-Zn rice varieties with Zn differentials of 8–10 and 10–12 μg g^{-1}, respectively, have made significant progress.

The Zn biofortification of rice is influenced by several environmental factors such as soil pH, soil composition, soil mineral status and availability, microbial populations, water management and fertiliser application. Understanding the GxE, and effectively addressing it through the development of more stable high-Zn rice genotypes targeted to different environments, along with water and fertiliser management options, is essential for the success of these varieties.

The challenge of mainstreaming in the conventional breeding is the need of molecular marker representing a major effect quantitative trait locus (QTL) along with genomic selections for minor alleles to 'fast-tract' trait introgression to all potential breeding lines in the pipeline. Population improvement approaches in combination with rapid genetic advance systems and high-throughput phenotyping and genotyping platforms are being explored to improve the genetic gain for grain Zn and yield in rice at IRRI.

Recently, the availability of more affordable genotyping services, whole-genome analysis can be performed for a higher number of breeding lines and subsequently used in genomic predictions and genomic selection for grain Zn and yield. In addition, the absorption and bioefficacy of the nutrients can be improved by enhancing the content of bioavailable minerals in the edible part of the staple crops, whilst improving the fibre content and reducing the anti-nutrients.

At IRRI, we are applying conventional and molecular breeding approaches, as well as transgenic approaches to develop high-Zn rice varieties. The pathway for development and dissemination of high-Zn rice varieties is depicted in Fig. 1.1.

Table 1.2 High-zinc rice varieties released under the HarvestPlus programme for commercial cultivation in Bangladesh, India and the Philippines

Variety	Season	Yield (t/ha)	Maturity (days)	Zn (μg g^{-1})	Country
NSICRc460	Dry and wet	4.0–5.0	119	19.6	Philippines
Chhattisgarh Zinc Rice 1	Dry and wet	4.5–5.0	110	22–24	India
DRR dhan-45	Wet	5.0	125	22.6	India
BRRI dhan 62	Aman	4.0–4.5	100	19.6	Bangladesh
BRRI dhan 64	Boro	6.0–7.0	145	24.6	Bangladesh
BRRI dhan 72	Aman	6.0–6.5	128	22.2	Bangladesh
BRRI dhan 74	Boro	7.0–8.3	147	24.2	Bangladesh

Table 1.3 Retention of zinc after cooking in different rice varieties produced either through conventional breeding or genetic engineering approaches

Germplasm	No. of samples	Times of washings	Method of cooking	Zn retention (%)	References
Philippine rice varieties	90	3	Cooked in test tubes	>85	Inabangan-Asilo et al. (2014)
Malaysian rice varieties	5	2–3	Pressure cooked	No significant reduction	Chapagai et al. (2017)
Colombian rice varieties	1	1–2	–	>79	Talsma et al. (2016)
Bangladesh rice varieties	15	2–3	Traditional cooking	>84	Ann et al. (2015)
Rice varieties from India	6	2–3	Different methods of cooking	No significant reduction	Bhandari (2013)
California rice varieties	3	–	Electric rice cooker and steamer	No reduction	Toma and Tabekhia (1979)
Transgenic rice lines	2	2	Cooked in test tubes	>90	Slamet-Loedin unpublished data

1.3.2 Genetic Engineering Approaches

The divalent nature of Zn, and hence its similarities with the Fe^{2+}, the related routes for acquisition and translocation within the rice plants and the utilisation of genes/enzymes in the generated constructs that promote the binding, absorption and/or translocation of Zn resulted in its subsequent increase during the iron biofortification efforts (Slamet-Loedin et al. 2015). A series of approaches have been identified for this purpose and are summarised in Fig. 1.1.

In a recent study, we showed that the Zn content of biofortified polished grains can be increased up to 2.7-fold compared to the wild type counterpart, reaching up to 45.7 $\mu g\ g^{-1}$, or added approximately 30 $\mu g\ g^{-1}$ Zn to the baseline of the popular cultivars (Trijatmiko et al. 2016). This increase fulfilled the nutritional dietary targets of both Fe and Zn biofortification under field conditions without yield penalty. Moreover, the performance of the in vitro $CaCO_2$ assay suggested that both the additional Fe and Zn were bioavailable. The increase in Zn content in polished grains of event NASFer-274 was mainly achieved due to the consecutive expression of rice nicotianamine synthase 2 (*OsNAS2*) gene, a precursor of the metal chelator nicotianamine (NA) (Kobayashi and Nishizawa 2012). Utilisation of the same gene (*OsNAS2*) or its isoforms from rice (such as *OsNAS1* and *OsNAS3*), barley (*HvNAS1*) and arabidopsis (*AtNAS1*) *in solo* or in conjunction with other genes resulted in

subsequent increase in the Zn content of biofortified rice grains (Higuchi et al. 2001; Masuda et al. 2009, 2012; Lee et al. 2009b, 2011; Wirth et al. 2009; Zheng et al. 2010; Johnson et al. 2011; Aung et al. 2013; Boonyaves et al. 2017; Singh et al. 2017).

In certain cases, ectopic or constitutive expression of *NAS* genes was combined with the barley nicotianamine aminotransferase (*NAAT*) gene (Masuda et al. 2008; Suzuki et al. 2008; Banakar et al. 2017). Both of these genes are involved in the biosynthetic pathway of mugineic acid (MA) family of phytosiderophores in graminaceous plants (Kobayashi and Nishizawa 2012). The rationale behind this approach was to further increase the production of MAs and subsequently the uptake and translocation of the metal ions such as Fe and Zn. Utilisation of this approach resulted in up to 2.2-fold increase in the Zn levels of polished rice grains (Banakar et al. 2017). However, this was a pot experiment, and hence, it needs to be validated under field condition.

Other transgenic approaches that was originally aimed to increase Fe but also increase grain Zn modestly included utilisation of ferritin genes from rice (Paul et al. 2012) or soybean (Vasconcelos et al. 2003; Qu et al. 2005; Paul et al. 2014), constitutive expression of the ferrous transporter *OsIRT1* (Lee and An 2009), the yellow stripe-like 15 (OsYSL15) (Lee et al. 2009), the bHLH Fe-related transcription factor gene *OsIRO2* in rice (Ogo et al. 2006), the mugineic acid family phytosiderophores 1 transporter (*TOM1*) from rice (Nozoye et al. 2011) and the vacuolar Fe transporters (OsVIT) (Zhang et al. 2012). In all these cases, the increase in the concentration of Zn in rice endosperm was insignificant compared to the wild type (Slamet-Loedin et al. 2015).

1.4 Factors Affecting Zinc Retention and Bioavailability

1.4.1 Effect of Polishing and Cooking on Zinc Retention

Most of the rice consumed is processed by dehulling, polishing, soaking, washing and cooking, which has an impact on the consumption levels of micronutrients such as Fe and Zn. Retention of a significant proportion of Zn in the polished and cooked rice is essential to provide health and nutritional benefits to the Zn-deficient populations (Oghbaei and Prakash 2016). The starchy endosperm or white rice is surrounded by pericarp and an aleurone layer known as the bran, which in turn is surrounded by a thick covering called husk (Champagne et al. 2004). During the process of dehulling, the husk is removed leaving the brown rice or caryopsis. The husk weighs about 20% of the total grain weight. The other by-products being removed during this process includes pericarp, nucellus, germ and the bran. Milling, on the other hand, removes the outer maternal tissue or the aleurone layer exposing the endosperm, resulting in polished or white rice. The degree of milling, abrasiveness and duration of milling influences the mineral retention. It is estimated that 2–10% of the weight of brown

rice is removed during milling resulting in the loss of most of the Fe and a significant portion (<40%) of the grain Zn (Juliano 2003; Rao et al. 2014; Ziarati and Azizi 2014; Talsma et al. 2016). It is also noteworthy that significant amounts of phytate (>80%), a major hindering factor for Zn absorption in human body, are also removed whilst polishing. A rat feeding study with milled rice of PSBRc 14 showed that the improved Zn/phytate ratio has significantly increased the bioavailability of Zn (Hunt et al. 2002).

Soaking and washing of polished rice before cooking to remove bran, dust and dirt from the food is a common practice in Asian households. However, during soaking and washing, some water-soluble nutrients are also removed unintentionally. There are reports showing a significant decrease in Fe, but a slight or negligible decrease in Zn concentration after washing of milled rice (Johnson et al. 2011; Kyriacou et al. 2014; Rao et al. 2014; Trijatmiko et al. 2016). Since Zn is embedded mostly in the endosperm, it is relatively stable even after soaking, washing and cooking.

Retention studies to evaluate the remaining amount of Fe and Zn in rice grains after processing are the first step to determine the nutritional target of biofortification programmes. Subsequently, it is essential to estimate the bioavailability of grain nutrients in human and its efficacy to improve the nutrient status in the human body. The method of cooking, as well as the food mixture, is very important for the absorption of Fe and Zn from the diet. In Table 1.2, a series of retention studies of zinc is presented after cooking in different rice varieties produced either through conventional breeding or genetic engineering approaches.

We studied the genetic variability for grain Zn in raw, washed and cooked rice samples at IRRI. We carried out Fe and Zn retention after milling, washing and cooking using high-Zn breeding lines developed at IRRI and also in milled rice purchased from different stores in Los Baños, Laguna and Philippines. Results showed that more than 85% of the Zn content of the raw rice is retained after three times of washing and cooking both in the market and advanced breeding lines, whereas most of the Fe was removed during these process (Ann et al. 2015). Similarly, in an advanced breeding line of upland high-Zn rice from Colombia, 79% of Zn was retained after polishing and cooking, whilst most of the phytate was removed (87%) (Talsma et al. 2016). In a set of Malaysian rice varieties, different methods of cooking did not significantly change the Zn content in the brown rice (Chapagai et al. 2017). Even if there were some minor variations in Zn retention in different rice varieties and different methods of cooking, there was no significant reduction in the overall Zn content (Mayer et al. 2007). However, a slight increase in Zn content after cooking has also been reported, which is mainly due to the release of Zn from the Zn–protein complexes upon heating, but this increase was not significant (Rao et al. 2014). Decanting water upon cooking rice was found to significantly decrease the minerals. There was no significant difference in phytate and Zn content among different rice products and rice varieties from Malaysia. The phytate/Zn ratio was <15 indicating good bioavailability of Zn even after cooking (Norhaizan and Nor Faizadatul Ain 2009).

In addition, it is interesting to note that the transgenic lines reported in Trijatmiko et al. (2016) showed a high retention efficiency (>90%) of grain Zn after cooking in

test tubes (Slamet-Loedin, unpublished data). Such evidence further suggests that the development of high iron and zinc-biofortified rice through transgenic approaches can result in improved retention efficiency for zinc.

1.4.2 Bioavailability of Zn in Human

Bioavailability refers to the portion of intake that can be absorbed into the blood system and utilised for physiological functions of the body. For Zn, it is determined by the following factors, including the Zn status of the individual, the total Zn content of the diet and the availability of soluble Zn from the diet's food components (Lönnerdal 2000). In cases where the Zn status in humans is lower than the physiological requirements, the solubility in the intestinal lumen determines its absorption. This is affected mainly by the Zn chemical form in the composite meals and the extent of its interaction with specific inhibitors and enhancers (Roohani et al. 2013).

The absorption of dietary Zn to a certain extent is affected by the human Zn status. A study on the long-term supplementation of Zn in healthy subjects showed a small increase in serum Zn values observed after 30 weeks of supplementation (Sandström et al. 1990). Moreover, a series of studies have demonstrated that providing individuals with Zn supplementation and subsequently lower the zinc diets enhances Zn absorption regardless of age group as homoeostatic mechanisms regulate both its absorption and retention (Istfan et al. 1983; Wada et al. 1985; August et al. 1989; Wang et al. 2017). The amount of zinc in the meal also affects the fraction of absorption (Lönnerdal 2000). An efficacy study among school-aged children in Thailand over a 6-month period showed that Zn supplementation increased linear growth in a population group with previous inadequate Zn intake (Rerksuppaphol and Rerksuppaphol 2017).

A variety of dietary factors can influence the absorption of Zn by the blood system in humans. Zinc in animal source foods such as shellfish, meats and eggs has relatively higher bioavailability because of the absence of zinc absorption inhibitors and the presence of zinc absorption enhancers, such as sulphur-containing amino acids. Phytic acid (inositol hexa- and penta-phosphate), as discussed later, is the primary dietary factor inhibiting the bioavailability of zinc (Hambidge et al. 2011). In the small intestine of rats, in vitro experiments have shown that zinc phytate is highly insoluble at their pH range and that addition of calcium to the medium exacerbated the production of an insoluble complex containing zinc, calcium and phytate (Oberleas et al. 1966). However, dietary calcium did not enhance the effects of phytate inhibition of zinc absorption in humans when they were exposed to conventional diets, indicating that calcium per se does not inhibit zinc absorption (Hunt and Beiseigel 2009).

Studies to determine how the nature of interaction between Fe and Zn affects the zinc bioavailability in humans (Solomons and Jacob 1981) showed that the interaction of inorganic zinc (in the form of zinc sulphate) with non-haem iron (in the form of ferrous sulphate) resulted in slight inhibition of zinc absorption when the Fe/Zn ratio

was 1:1 and substantial inhibition when the Fe/Zn ratio was 2:1 or 3:1. However, supplementation of non-haem Fe in the form of haem chloride was found to have no effect on zinc absorption even at 3:1 Fe/Zn ratio. Similarly, absorption of 'organic' zinc from Atlantic oysters in the presence of iron did not significantly affect the zinc absorption (Solomons and Jacob 1981). When a dosage of zinc similar to that obtained from composite meals was used, it was found that the effect of iron on zinc absorption is evident only at very high levels of iron to zinc and in aquatic solution, further suggesting that iron fortification will not have an impact on zinc absorption (Lönnerdal 2000). This has also been validated when the zinc absorption was measured after consumption of both adults and infants with iron-fortified food (Davidson et al. 1995; Fairweather-Tait et al. 1995).

The positive influence of protein intake and zinc absorption has been well-documented (Sandström et al. 1989; Lönnerdal 2000). In particular, the ingestion of animal proteins can significantly improve the bioavailability of Zn, possibly because the released amino acids facilitate zinc solubility (Lönnerdal 2000). On the contrary, the absorption of zinc in plant-based foods is hindered by the presence of phytate in plant cells (Egli et al. 2004), often leading to a reduction of the zinc uptake and bioavailability, particularly in vegetarian diets. However, no adverse health effects from lower zinc absorption have been demonstrated (Hunt 2003). Nevertheless, interaction or binding of zinc to soluble ligands or chelators improve the zinc solubility as such, resulting in a positive effect on zinc absorption (Lönnerdal 2000).

1.4.3 The Role of Phytate in Bioavailability of Zinc

A number of factors affect the bioavailability of zinc for human absorption including phytic acid (PA) and its ratio to other components in the food matrix, such as the phenolic compounds and fibre content (Bohn et al. 2008). However, PA is the major contributing factor. Phytic acid (inositol hexa-phosphate) is an unstable compound composed by the attachment of six phosphate ester groups in an inositol ring that is considered to influence the absorption of zinc by the human body. When interacting with metals, like magnesium (Mg^{2+}), calcium (Ca^{2+}) or potassium (K^{+}), it produces salts after covalent bond formation and subsequent neutralisation of its anions (Lopez et al. 2002). Phytate is the primary storage form for phosphorus in many plant tissues including the bran (aleurone layer) of cereals and its content varies from 0.06 to 2.22%, with polished rice grains containing the lowest amount (Reddy 2001; Gibson et al. 2010). Other parts of plants including roots, tubers, most of the leafy tissues (e.g. vegetables) and fruits contain minimal amounts of phytate, whereas animal foods contain none (Gibson et al. 2010).

Metal ions, particularly Zn, Fe and Ca, are chelated by phytic acid but not Cu (Egli et al. 2004), forming insoluble complexes inside the gastrointestinal tract that are unavailable for digestion or absorption by humans because of the absence of intestinal phytase enzymes(Iqbal et al. 1994; Hambidge et al. 2011). The inhibitory effects of phytic acid on zinc absorption can be predicted by the molar ratios of

phytate: zinc in the diet. According to the World Health Organization (WHO) and the International Zn Nutrition Consultative Group (IZiNCG), molar ratios in excess of 15:1 or 18:1, respectively, have been linked to sub-optimal zinc status in humans (International Zinc Nutrition Consultative Group (IZiNCG) 2004; Allen et al. 2006). Consequently, the bioavailability of these elements can be improved by dephytinisation, a process related to the reduction in phytate concentrations in certain plant tissues (Egli et al. 2004; Gibson et al. 2010). In cereal grains, phytate is mainly localised in the aleurone layer (i.e. brown rice) and embryo parts, whilst minuscule amounts have been detected in the endosperm (Lehrfeld and Wu 1991; Prom-u-thai et al. 2008; Persson et al. 2009; Jaksomsak et al. 2014; Saenchai et al. 2016). Moreover, speciation and localisation studies on cereal grains have revealed the association of zinc with proteins instead of phytate (Persson et al. 2009, 2016; Kutman et al. 2010; Lombi et al. 2011; Kyriacou et al. 2014), therefore indicating its potential bioavailability.

In recent years, several attempts (including genetic improvement, as well as fermentation, soaking, germination and phytase enzymatic treatment of grains) have been followed to reduce the phytic acid content in cereal grains and hence improve their nutritional value (Gupta et al. 2015). However, the phytic acid beyond its role in phosphorus storage is also a very important signalling molecule involved in several regulatory processes; consequently, generation of low phytic acid mutants resulted in the production of plants with different negative pleiotropic effects (Sparvoli and Cominelli 2015). Nevertheless, transgenic technologies in rice have proven more effective in generating low phytic acid plants whilst overcoming these effects (Ali et al. 2013; Perera et al. 2018).

1.5 Deployment Path and Potential Impact of Biofortification Zinc

Achieving the most cost-effective and large-scale impact of biofortification will require the combination of all the available approaches for the improvement of nutrient deficiency status (e.g. biofortification, supplementation and fortification). However, biofortification has key advantages because of its ability to reach rural populations and their long-term cost-effectiveness (Bouis and Saltzman 2017). In order to be successful, biofortification requires robust products from breeders, the establishment of nutrient efficacy that was discussed earlier and certainly farmer and consumer adoption (Meenakshi 2009). To calculate the cost-effectiveness of biofortification as a nutrition intervention, HarvestPlus used the disability-adjusted life years (DALYs) framework. This approach captures both morbidity and mortality outcomes in a single measure and is often used in health literature (Organization, no date).

In Bangladesh, consumption of rice reaches 99%, and many households still grow rice (38%) and Zn biofortification increased the discounted cost per DALY saved from

$12 to $32 (Birol et al. 2014). The preliminary results from a recent *ex ante* cost-effectiveness analysis on Zn and vitamin A crops indicated that biofortification can be rated as a 'very cost-effective' approach according the World Health Organization's CHOICE (Choosing Interventions that are Cost-Effective) Working Group, since the overall costs are significantly lower to the per capita income in developing countries, which ranges from US$365 in the Democratic Republic of Congo (DRC) to US$3843 in India (Birol et al. 2014). Even though biofortification requires a high upfront investment, it has the potential to eventually be self-sustaining and sustainable (Bouis and Saltzman 2017).

The value chain in rice covers from seed producers, farmers, miller and consumers. At the crucial stage-gate, a simple but effective quality control system to measure the level of micronutrient prior to the market distribution will need to be implemented to ensure the effectiveness of this intervention.

Learning from the lessons of research-to-implementation of other biofortified crops (sweet potato and maize) (Tanumihardjo et al. 2017), the deployment of Zn-biofortified rice will require a multi-stakeholder and multi-stage effort involving the agriculture, trade, health and nutrition sectors. Ensuring the agronomic productivity (including crop diffusion and farmer-to-farmer seed sharing) of the rice variety will be crucial to its sustainability. Generating consumer acceptance and preference for Zn-biofortified rice versus the standard rice will enhance impact. Of particular importance here is the creation of demand and promotion of access to Zn-biofortified rice among the populations that are likely to benefit from the additional Zn. As identified early in this chapter, these are the poor and rural communities who are not easily reached by other interventions that address Zn deficiency. In addition, market strategies to provide access to Zn-deficient urban populations need to be developed. The involvement of local and national policymakers and stakeholders, in both the private and public sectors, can facilitate scaling up and integration into the mainstream rice value chain (Bouis and Saltzman 2017).

1.6 Conclusions

A number of rice varieties with elevated levels of Zn have been released. The advantage of conventional or marker-assisted breeding lowers the hurdles in the regulatory pathway to release the variety and there is no issue on public acceptance. However, achieving the Zn nutritional target of 28 $\mu g\ g^{-1}$ in polished grains over multiple environments remains challenging. In addition, until now there has not been identified any major effect QTL for grain Zn, whilst the availability of molecular markers is necessary to allow marker-assisted breeding for Zn. The availability of marker(s) will ease the breeding to multiple genetic backgrounds relevant to different geographical locations.

The advantage of the biofortified product through GM approaches is that the level of grain Zn achieved over surpasses the nutritional target levels for zinc, and the transgene itself can serve as the marker for subsequent smooth introgression

to multiple varieties. Moreover, the combination with the evolving genome editing technology will enable in the future pyramiding of multiple nutrition and other traits (e.g. agronomic) in one locus. The GM approach has the disadvantage of potential resistance affecting public acceptance, whilst this might alter in favour because of the robust food and feed safety analysis. It is undoubtedly preferable to have rice enriched with micronutrients as the main source of caloric intake food, rather than consuming an 'empty calorie' option. Supported with a rigorous deployment plan for wider market adoption and simultaneous promotion of the health benefits, zinc-biofortified rice may provide the carrier for improving and enhancing micronutrient sufficiency in rice-consuming populations.

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Competing Interest Authors declare that they do not have any competing interest.

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Chapter 2
All Roads Leading to: Iron Phytofortification

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Abstract Enhancing the nutritional quality of food crops is an arena of research that is receiving much footfall among the scientific fraternity, owing to the heavy dependence of humans on plant-based diets. These diets often lack essential micronutrients like iron, thus compromising health and productivity, especially among women and children from impoverished countries. Additionally, although iron is abundant in soil, the calcareous nature of soil renders it unavailable to the plant. This has aggravated the problem, as the bioavailability of iron from staple plant foods is already low in humans. Thus, increasing iron content in the edible portions of the plant may help ameliorate this deficit. Toward this, the past decade has witnessed extensive focus on understanding the mechanisms of iron uptake and redistribution in plants. This understanding has afforded greater insight into altering the existing strategies to achieve this goal. Approaches such as crop and mutation breeding, as well as transgenic technologies, have been used for the introduction of useful traits via genome manipulation. Also, a newly emergent area of study concerns the interaction of rhizosphere microbiota with plant roots and their effects on iron acquisition by plants. In this chapter, we have attempted to summarize the impact of associated microbiota in iron acquisition in addition to the conventional and transgenic efforts toward phytofortification for the alleviation of iron deficiency anemia in the global populace, particularly the affected strata.

Keywords Biofortification · Phytofortification · Iron · Anemia · Plant growth promoting rhizobacteria (PGPR) · **V**olatile organic compounds (VOC) · Induced systemic resistance (ISR) · Crop improvement · Nutrition

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2.1 Introduction

2.1.1 The Need for Crop Improvement: An Overview

Nutrient balance is crucial to the survival and health of all organisms throughout the food chain. With the world population slated to ascend in the years to come, land space, soil fertility, water accessibility, and hence nutrient availability are susceptible to degradation due to industrial activity and overcultivation. Thus, the pressing need of the hour is the development of technologies for the production of water-efficient as well as nutrient-enriched crops to continue agriculture in a sustainable manner, without excessive reliance on environmentally threatening fertilizers and pesticides (Tilman 1999). In this regard, the element iron is by far one of the most abundant in the earth's crust and an essential requirement for organisms. Plants forming the largest trophic food base and the source of nutrition for higher organisms through photosynthate production are consequently a major consumer of this element. Ironically, however, iron is poorly available for capture and consumption by plants due to its tendency for oxidation and precipitation as hydroxides, siderites, and phosphates, rendering it insoluble and thereby unavailable (Lindsay and Schwab 1982; Guerinot and Yi 1994). A number of studies have together elucidated the mechanistic basis for iron uptake and distribution throughout the plant body.

The level of iron in the plant biomass is ultimately partitioned into the higher trophic levels through the consumption of vegetarian foods. However, the utilizable amount depends on the bioavailable fraction in the plant, as plant iron is generally complexed with the anti-nutrient phytate, reducing its availability (Drakakaki et al. 2005). In contrast, iron from animal sources is readily bioavailable as heme-Fe complex, underscoring the importance of a diversified diet inclusive of both vegetable and animal sources to achieve healthy balanced nutrition (Layrisse et al. 1969; Hurrell and Egli 2010). This is a concern for primarily vegetarian populations as well as the poor, socioeconomically disadvantaged and other marginalized people who are unable to afford such a diet, predisposing them to micronutrient deficiencies. Iron deficiency, in particular, is a grave threat to infants, children, young girls, and pregnant women. This manifests in delayed and abnormal growth and development of the foetus, poor cognition and performance at work, fatigue and lowered immunity as well as poor absorption of other nutrients (Walter et al. 1989; Allen 2000; Zimmermann et al. 2000; Hess et al. 2002; Beard 2003). This has necessitated the institution of several remedial measures, including iron supplementation (Bruner et al. 1996) and food fortification (Zimmermann et al. 2003; Zlotkin et al. 2003; Zimmermann and Hurrell 2007; Andersson et al. 2008). These measures although in place, have not met with much success owing to various reasons, which include: variable consumer demands and acceptance, undesirable organoleptic changes in fortified food, physiological disturbances, and socioeconomic-political considerations (Galloway and McGuire 1994; Hurrell 2002; Iannotti et al. 2006; Baltussen et al. 2004; Hoppe et al. 2006). Another source of concern is the heavy dependence of the poor on the so-called "staple" crops such as cassava, wheat, and rice, which lack in major

micronutrients (Gegios et al. 2010). Since staple crops are those that are largely cultivated in a given geographical area and preferred due to socioeconomic reasons, improving them with respect to their nutritional quality is an option worth exploring. Thus, a third method involves biofortification of crops like cassava, banana, rice, wheat, maize, beans, and millets to improve their endogenous iron content. Biofortification is a means to enhance the intrinsic nutritional content and bioavailability in the plant, such as that of minerals, protein, and beneficial bioactive compounds through genetic or agronomic technologies (White and Broadley 2009). Currently, the major target micronutrients focused upon the development of biofortified crops are iron (Haas et al. 2005; Ihemere et al. 2012), zinc (Velu et al. 2014; Trijatmiko et al. 2016), vitamin A (La Frano et al. 2013), and iodine as under the aegis of the HarvestPlus program (Pfeiffer and McClafferty 2007). This chapter aims to provide only a bird's eye-view on iron uptake and redistribution in plants and the strategies already exploited toward iron biofortification. In this regard, the readers are directed toward previously published reviews described earlier for a detailed description of the same (Patel et al. 2016). Apart from traditional breeding and genetic engineering, interest has also been generated in the interactions of plant roots with rhizosphere microflora and their influence on plant nutrition, as it is well known that plants are dependent on beneficial soil organisms for various mineral requirements. For example, mycorrhiza like *Glomus*, help in phosphorus uptake, when associated with roots of gymnosperm species (Zandavalli et al. 2004). Similarly, association of *rhizobia* with leguminous plants have been long since known to enhance the nitrogen-uptake abilities of the plant and thereby its growth and development. It is well known that certain microbes secrete siderophores for iron acquisition from the soil and thus possibly compete for iron with plant roots. Thus, the exploitation of these microbes for the enrichment of plant iron reserves is an arena with immense potential, in addition to standard agronomic practices such as application of fertilizers to the soil (Aciksoz et al. 2011). A broad understanding of the roles played by these organisms with respect to iron has been explored in some depth and presented in this chapter.

2.2 Plant Uptake and Distribution: An Overview

2.2.1 Plant Iron Acquisition: Endogenous Dramatis Personae

To meet the cellular demands under variable soil conditions, predominantly dictated by pH, plants have evolved two strategies for efficient iron acquisition, simultaneously preventing iron overload. On the basis of key players responding to soil iron status, plants are broadly classified as Strategy I and II, both of which have been reviewed extensively (Marschner et al. 1986; Kobayashi and Nishizawa 2012). The former relies on soil acidification with iron reduction and the latter on chelation, respectively. Strategy I plants are dicots and non-graminaceous monocots, which extrude

protons into the rhizosphere via the AHA pump (Santi and Schmidt 2009), followed by FRO2-mediated reduction of the mobilized iron into ferrous form (Robinson et al. 1999) which is subsequently imported into the root through the IRT1 transporter (Korshunova et al. 1999; Vert et al. 2002). Strategy I plants have also been shown to secrete phenolic compounds such as flavonoids by red clover (Jin et al. 2007), protocatechuic acid and caffeic acid by rice (Ishimaru et al. 2011; Bashir et al. 2011a), and coumarin derivatives (Fourcroy et al. 2014) into the apoplastic space to solubilize precipitated iron. The strategy I response is transcriptionally regulated in the root epidermal cells, by the FIT/FRU/FER transcription factor (TF) (Ling et al. 2002; Yuan et al. 2005) which interacts with bHLH038 and bHLH039to induce the IRON-REGULATED TRANSPORTER 1 (*IRT1*) and ferric chelate reductase (*FRO2*) expression under iron deficiency (Yuan et al. 2008). Acting independent of FIT and independently induced under iron deficiency, are bHLH100 and bHLH101. These TFs are also essential for a robust iron deficiency response as they affect a subset of genes not under the purview of FIT, but which are required for iron remobilization and utilization within the plant (Wang et al. 2007; Sivitz et al. 2012). Another circuit operative in the root pericycle is the POPEYE (PYE)-PYE-LIKE (PYEL)-BRUTUS (BTS) network. The bHLH TFs POPEYE and PYEL (which include bHLH104, bHLHL115, and IAA-LEUCINE RESISTANT ILR3) are positive regulators (Rampey et al. 2006; Long et al. 2010) while BRUTUS negatively regulates the iron deficiency response by 26S proteasomal degradation of PYEL proteins (Selote et al. 2015). A connection between these two apparently distinct regulatory domains was recently discovered as bHLH104, bHLH115, and bHLH34 were shown to directly activate the bHLH38/39/100/101 quartet collectively known as the bHLH subgroup 1b genes (Wang et al. 2013; Li et al. 2016b; Liang et al. 2017). Furthermore, bHLH104 interacts with the PYEL ILR3 to activate expression of *PYE* and the subgroup 1b genes, thus establishing coordination in the signaling cascade responding to iron deficiency (Zhang et al. 2015). This network influences the root system architecture, iron storage, iron mobilization, and transport between tissues and iron sequestration in vacuoles under iron deficiency (Long et al. 2010).

Contrastingly, strategy II functions in graminaceous plants such as wheat and maize and utilizes secreted phytosiderophores to chelate ferric irons directly from the soil (Römheld and Marschner 1986). The methionine cycle synthesizes S-adenosyl methionine (SAM), which is trimerized by nicotianamine synthase (NAS) to produce a molecule of nicotianamine (NA). This NA is subsequently converted into different species-specific mugineic acids (MA) also called phytosiderophores (PS) such as deoxy-MA in rice and epi-hydroxy MA in barley (Nakanishi et al. 2000; Kobayashi et al. 2005). These non-proteinic negatively charged amino acids are extruded into the rhizosphere via the TRANSPORTER-OF-MA 1 (TOM1) transporter (Nozoye et al. 2011), where they are able to free the ferric iron bound to negatively charged soil compounds and make it available to the plant. The Fe^{+3}-PS complexes are then internalized through YELLOW STRIPE 1/YS1-LIKE (YS1/YSL) proteins in the root epidermal membrane (Curie et al. 2001). The strategy II response is coordinately controlled and fine-tuned through positive and negative regulation to allow for iron uptake under deficiency and distribution through the plant while preventing iron

overload. Positive regulation is mediated by the bHLH protein IRO2, a transcriptional activator of genes for mugineic acid synthesis (Ogo et al. 2007). The Bhlh transcription factor IDEF1 belonging to the ABI3/VP1 family senses iron status through direct binding of the ion and activates IRO2 by binding to the IDE1 *cis*-element in the promoter of IRO2 as well as MA synthesis pathway genes (Kobayashi et al. 2007, 2012) to activate genes for both early and late responses to iron deficiency (Kobayashi et al. 2009). Another transcription activator IDEF2 is a NAC (NAM, ATAF and CUC) family member, which activates several iron deficiency responsive genes such as rice YSL2 through binding to the IDE2 sequence in the promoter (Ogo et al. 2008). Negative regulation of strategy II is imposed by rice IRO3, which is specifically induced by iron deficiency and targets IRT1, NAS, and YSL genes under iron starvation, probably to limit excessive iron intake and control input into the energy-intensive MA synthesis pathway (Zheng et al. 2010b).

2.2.2 Iron Transport and Distribution

Though iron is taken up through differing strategies, it follows a largely conserved path into the aerial tissues, aided by protein homologs for cellular utilization, storage, and transport to the sink tissues. Iron intake in strategy I plants is in the ferrous form, while that in strategy II plants is as ferric bound to PS. In the root, ferrous iron enters the xylem through an unknown transporter protein, while ferric ions are imported into the xylem bound to citrate which is itself effluxed through the multidrug and toxin efflux transporter (FRD3) in *arabidopsis* and its rice homolog, the Ferric Reductase Defective-Like transporter (FRDL1) (Durrett et al. 2007; Yokosho et al. 2009). FRD3 is also essential for iron nutrition of tissues having no symplastic connections, such as the male gametophyte and seeds, as citrate mobilizes apoplastic iron for the utilization by these tissues (Roschzttardtz et al. 2011). The transpiration stream helps the movement of iron through the xylem into the aerial portions. Further, the iron is unloaded into the phloem for transport into the sink regions, via the YSL proteins, which also show organ-specific expression such as that in seeds, pollen tubes, and lamina joints. In rice, *Os*YSL2/15 and in *arabidopsis*, *At*YSL1/3, mobilize iron into the inflorescence and seed in complexes with NA (Koike et al. 2004; Inoue et al. 2009; Jean et al. 2005; Waters et al. 2006; Chu et al. 2010). Rice YSL18 transports Fe^{+3}-DMA complexes into reproductive organs and vegetative parts such as lamina joints and leaf sheaths as well as parenchyma of crown roots (Aoyama et al. 2009). In *arabidopsis*, the Oligo Peptide Transporter (*At*OPT3) located in the phloem of leaf minor veins and stem nodes, pollen and developing embryos mediates iron transport into seeds and further redistribution from leaves to other sink tissues via phloem (Stacey et al. 2002, 2008). While complete disruption of OPT3 is embryo-lethal, non-lethal knockdown mutants show deranged shoot signaling of iron status to the root (Zhai et al. 2014). Interestingly, OPT3 blocks the translocation of cadmium into sensitive sink tissues while selectively partitioning iron into them, underscoring

its importance in precluding the translocation of toxic heavy metals taken up due to IRT1 activity under iron deficiency (Zhai et al. 2014; Mendoza-Cózatl et al. 2014).

Cellular iron is diverted toward the chloroplast for photosynthesis and the mitochondrion for respiration. For intracellular transport into the chloroplast, Fe^{+3} is reduced to Fe^{+2} by FRO7 (Jeong et al. 2008) and then mobilized into it by PERMEASE IN CHLOROPLASTS 1 (PIC1) located in the chloroplast membrane and essential for chloroplast development (Duy et al. 2007). Iron entry into the mitochondrion may involve the reduction of ferric iron via the FRO3 and FRO8 homologs of ferric chelate reductase in *Arabidopsis* (Jeong and Connolly 2009). The Mitochondrial Iron Transporter (MIT) (Bashir et al. 2011b) mediates mitochondrial import of iron, whereas the mitochondrial ATP-binding cassette transporter (*ATM3)* exports iron into the cytosol for cytosolic Fe-S cluster assembly (Zuo et al. 2017). In both the chloroplast and mitochondrion, iron is sequestered by ferritin as precipitated iron phosphates within the 24-mer-protein core (Petit et al. 2001, Zancani et al. 2004). In the mitochondrion, an additional protein frataxin, a Fe-S cluster-containing protein, also maintains iron homeostasis, until usage and remobilization for specific cellular needs (Busi et al. 2004, 2006). Vacuolar iron import is mediated through root epidermal FERROPORTIN 2 (FPN2), which also imports cobalt (Morrissey et al. 2009) and the VACUOLAR IRON TRANSPORTER 1 (VIT1) (Kim et al. 2006). Once inside the vacuole, iron is either complexed by NA or phytate for safe storage till required. The Natural Resistance-Associated Macrophage Proteins (NRAMP3 and NRAMP4) efflux iron into the cytosol to meet cellular metabolic demands (Lanquar et al. 2005).

Hormonal orchestration of the iron homeostatic pathway is mediated by auxin, nitric oxide (NO), ethylene, cytokinin, and jasmonate, which integrate plant metabolic status with the iron deficiency response. Herein, a network of sucrose, nitric oxide, and auxin is established. Iron deficiency induces endogenous accumulation of sucrose, which enhances auxin signaling (Lin et al. 2016). Auxin mediates root system architectural changes in response to Fe deficiency in a gradient dependent manner, as localized iron around the root strongly induced lateral root formation (Giehl et al. 2012). Auxin signaling also activates root NO production, which in turn stabilizes the FIT protein and upregulates IRT1 and FRO activity for increased iron uptake (Chen et al. 2010). Ethylene also induces the iron deficiency response via the interaction of EIN1/EIL3 with FIT, again by possibly stabilizing it against proteasomal mediated degradation (Lingam et al. 2011). On the other hand, cytokinins and jasmonate negatively regulate IRT1 and FRO2 through a FIT-independent manner. Cytokinins inhibit primary root growth under Fe deficiency, indicating coordination of plant growth with Fe requirements, while jasmonate suppresses IRT1 and FRO2 transcription but not their iron-inducibility, suggesting fine-tuning of iron homeostasis (Séguéla et al. 2008; Maurer et al. 2011). These observations suggest that the hormonal regulation of iron homeostasis serves to balance the uptake of iron with possible toxicity arising from the non-specific nature of metal uptake by IRT1 in Strategy I plants.

2.3 The Road Travelled so Far: Through Breeding and Genetic Engineering

Both crop breeding and genetic engineering have been explored for iron enrichment in food crops. The relative advantages of each over the other vary with the crop under consideration as wide variability exists in the amenability of plants toward transformation and in the germplasm base for iron content. This precludes some crops from manipulation by one strategy but opens avenues to utilize the other. Breeding for high iron content in seed and edible parts of the plant requires the existence of a sufficiently large genetic variability in iron content among parent lines (Sperotto et al. 2012) as well as minimal linkage drag. Selection for and stabilization of such a trait is a long-duration process and there may be unfavorable interactions with other traits of economic importance. However, breeding does not require prior knowledge of the loci involved, thus enabling the generation of high iron plants, which further need to be validated through marker-assisted technology. The release of varieties is also not hampered by regulatory constraints as it does not involve the usage of any foreign elements. Transgenic technology toward biofortification, on the other hand, necessitates prior knowledge of individual genes contributing to iron uptake, transport, and storage as well as the potential synergistic or antagonistic interactions between them. Furthermore, a gamut of regulatory approvals and safety tests are needed before the promising event is released for public consumption, as public acceptance of this technology is still low. However, it is useful to elucidate the functions of genes and offers options to stack genes for desirable traits and modify undesirable traits by RNAi silencing (Garg et al. 2018).

2.3.1 Iron Biofortification Through Classical Crop Breeding: Varieties Released

Although the timelines for the development of biofortified varieties through conventional breeding are significantly longer, the process holds hope for millions across the globe. As mentioned before, the regulatory hurdles for release of a variety developed through this method are minimal over the ones developed through transgenic technology. Crop improvement through conventional breeding requires the identification of elite genotypes in the germplasm. Past and current efforts have produced iron-fortified varieties of various crops notably wheat, rice, beans, lentils, cowpea, potato, sorghum, and millets. An aromatic rice variety "IR68144-3B-2-2-3" was developed to contain ~21 mg/kg of iron in the unpolished grain. This amount was almost twofold higher than the typical 12 mg/kg iron content found in common rice. The variety was also found to be tolerant to rice tungro virus and displayed better grain qualities (Gregorio et al. 2000). Wheat-Zhongmai 175 and Wheat-Zhongyou 9507 contain 30–44.5 and 34.9–57.8 ppm of iron, respectively. The polished rice variety Zhongguangxiang 1 contains 7 ppm of iron. Additionally, these varieties displayed increased resistance

and yield and were released in China in the years 2001–2010 (HarvestPlus 2014). Discovery of high-iron genotypes of beans led to the development of "bush" and "climber" varieties into high-iron elite varieties with 21–50 ppm of iron. The developed beans also displayed increased resistance to Ascochyta blight (Anthracnose). These developed varieties were then subsequently released in Rwanda in the years 2010–2013 (HarvestPlus 2014). Various varieties of lentils from Bangladesh, India, and Nepal were fortified with iron as the primary mineral target and zinc as secondary mineral target. Upon multi-location trials, L4704 with more than 85 ppm of iron and 74 ppm of zinc in India was released in 2012. In Nepal and in Bangladesh, ILL7723 and Barimasur-7 with more than 43 ppm and 41 ppm of iron, respectively, were released in 2013 (HarvestPlus 2014). Similarly, Irish potatoes and sorghum containing high iron levels have been developed for Rwanda-Ethopia and India, respectively. In India, cowpea genotypes (Pant Lobia-1, 2, 3 and 4) with iron content ranging from 51 to 100 ppm were released in the years 2008–2014. These also had higher content of zinc and were resistant to multiple diseases. Pant Lobia-1 and 2 were found to be early maturing and their seeds have now been made available to the farmers for local production (HarvestPlus 2014). Five bean varieties; NAROBEAN 1, 2, 3 4C and 5C which were found to be an excellent source of iron and were also drought resilient, were produced under the aegis of the HarvestPlus program (Nantongo 2016). Also, biofortified pearl millet "Dhanashakti" with 71 mg/kg iron and 40 mg/kg zinc was developed and released in India in addition to a high-iron pearl millet hybrid "ICMH 1201" which has 75 mg/kg iron, 40 mg/kg zinc and 30% higher grain yield than "Dhanashakti" (ICRISAT 2016). Recently, efforts have been made to identify the chromosome loci associated with the observed increase in the mineral content. Maize kernel iron and zinc concentrations in the germplasm were estimated, and the corresponding genomic regions were identified through GWAS. The genomic regions were further validated in bi-parental populations (Hindu et al. 2018).

Clinical trials using iron-fortified pearl millet were conducted for six months on teenagers from the Maharashtra state of India (Finkelstein et al. 2015) and Indian teenage girls (Beer et al. 2014). They revealed improved blood iron parameters such as hemoglobin and proved to be efficacious in mitigating iron deficiency anemia. In another clinical trial, 135 children fed with iron-fortified pearl millet reported increased physical performance in terms of maximal oxygen uptake in comparison with the control group who were fed non-fortified pearl millet. Also, the study revealed improved iron status in terms of serum transferrin receptor values in the test group compared to the control group (Pompano et al. 2013). Similarly, a trial using iron biofortified beans was conducted on 195 Rwandan women between the ages of 18–27 years. After 128 days of the trial, the test group fed with iron biofortified beans showed a significant increase in their hemoglobin and other blood parameters related to iron status compared to the control group who were fed control beans (Haas et al. 2016). These trials point out the many success stories of conventional breeding programs. However, for important staples deficient in iron for which iron-rich genotypes are scarce or for plants which are unamenable to cross pollination, transgenic technology has provided significant results, as discussed below.

2.3.2 Iron Biofortification Through Genetic Engineering

With regard to iron biofortification through transgenic means, the overarching theme has been the utilization of iron uptake, transport and sequestration-related genes in grain/edible part of the plants to allow for the enhanced translocation of iron through the plant, coupled with safe storage. Accordingly, at iron uptake level, the root iron transporter IRT1 has been overexpressed either alone as in rice (Lee and An 2009; Tan et al. 2015) or in conjunction with arabidopsis ferritin, common bean *NAS* and phytase (NFP lines), under a seed-specific nodulin promoter (Boonyaves et al. 2016). The latter strategy enhanced iron content above that of only NFP plants. However, a caveat was the increased sensitivity of IRT-overexpressing lines to cadmium and excess zinc due to the poor specificity of this transporter (Lee and An 2009). Other attractive candidates for iron biofortification include ferritin and NAS—the former because it can store up to 4500 atoms of iron in the protein core without associated toxicity and the latter because of its proven role in the translocation of iron through the plant. A brief overview of the same has been presented below.

Several groups have reported an increase in iron content in vegetative and/or grain parts of plants overexpressing ferritin either under constitutive or seed-specific promoters (Goto et al. 1999; Van Wuytswinkel et al. 1999; Drakakaki et al. 2000; Vasconcelos et al. 2003; Drakakaki et al. 2005; Paul et al. 2012; Masuda et al. 2013). This has been attributed primarily to the creation of an internal pseudo-iron deficient state in the plant through imbalance of steady-state iron concentrations due to over-sequestration by ferritin. Availability of iron for the translocation to needy organs is therefore impaired, thus activating iron uptake and translocation mechanisms (Van Wuytswinkel et al. 1999). Thus, FRO2 activity and proton secretion into the rhizosphere increased in tobacco plants overexpressing ferritin under a constitutive promoter (Vansuyt et al. 2003). In other cases, internal iron homeostasis appears to be disturbed by ferritin overproduction in a particular organ. Overexpressing soybean ferritin using a seed-specific promoter in a maize line with low phytate content, enhanced seed iron and its bioavailability, but disturbed iron distribution between tissues (Qu et al. 2005; Aluru et al. 2011). The maize leaf *YS1* and *FRD3* genes, involved in iron-chelate transport and citrate efflux, respectively, as well as nicotianamine synthase (*NAS3*), were induced in these plants, thereby increasing the mobilization of iron-NA from leaves to seed and decreasing leaf iron levels. Furthermore, both endogenous maize ferritin genes were suppressed in leaves to facilitate iron export. In contrast, mugineic acid production genes *NAAT1* and *DMAS* also showed altered expression in leaf and root, with *NAAT* downregulated in root but induced in leaf and *DMAS* strongly repressed in leaf, indicating that root uptake of iron was not increased and that higher ferritin levels may not parallel concomitant increase in root iron. On the contrary, Kanobe et al. (2013) showed an increase in root *Zm*NAAT1 in soybean ferritin-expressing maize, but no significant alterations in the NAS gene family expression, which was attributed to genetic background difference. They also reported an increase in the 4Fe-4S-containing ferredoxin genes, possibly affecting electron transport and iron homeostasis. Recently, a banana

ferritin *MusaFer1* was found to increase leaf and root iron content by twofold in leaf and threefold in roots, when expressed under the constitutive *Zm*Ubi promoter. The gene also conferred oxidative stress tolerance on the transgenic plants relative to the controls owing to the sequestration of reactive iron in inert form in the ferritin core (Yadav et al. 2017).

Due to the limitations observed in the accumulation of iron by overexpressing ferritin alone (Vansuyt et al. 2000; Qu et al. 2005) and the discovery that checkpoints for iron loading in seeds regulate ferritin stability and thus iron accumulation (Ravet et al. 2009), attempts were made to facilitate increased uptake, translocation and remobilization as well as storage, along with sequestration of iron. With the influx of basic research coupled with "OMICS" data, better insights on iron homeostasis pathway have been established over the years, allowing for a more "informed" selection of genes. Co-expression of FROs and ferritins has been suggested to facilitate more iron acquisition in comparison with the overexpression of single genes (Goto et al. 1999), together with storage in a safe form. Similarly, combinations of ferritin with NAS (Wirth et al. 2009), with NAS and YSL2 (Masuda et al. 2012), with NAS1, IDS3, and NAAT (Masuda et al. 2013), with IRT1 and NAS (Boonyaves et al. 2016) and with NAS and NRAMP3 (Wu et al. 2019) were generated. Placing the ferritin gene under a seed/endosperm-specific promoter and employing the above combinations yielded increases in seed iron content over that observed for ferritin overexpression alone. The levels of zinc were also increased in these plants due to the ability of NA to chelate this metal. Also, no significant undesirable alterations in growth and yield were noted in these transgenic plants. In addition to these measures, the expression of fungal phytase to degrade the anti-nutrient phytic acid enhanced iron bioavailability (Drakakaki et al. 2005, Aluru et al. 2011). Ferritin was isolated from *Phaseolus vulgaris* and its overexpression in Japonica rice variety Taipei 309 under the glutelin promoter yielded a twofold increase in transformed rice grains over controls. Additionally, to increase the bioavailability of iron, a heat-tolerant phytase isolated from *Aspergillus fumigatus* was overexpressed and its activity in the transgenic lines was 130-fold higher than the wild type. Further, a sevenfold increase was found in the content of cysteine residues which are considered as enhancers of non-heme form of iron (Lucca et al. 2001). Taken together, the synergetic "push–pull" mechanism obtained by the use of these constructs, led to effective enhancement of the iron and zinc levels in a safe manner, with potential applications in biofortification (Zielińska-Dawidziak 2015).

As described above, exclusive overexpression of transporters or storage genes is not sufficient to increase iron content. One of the reasons is that iron is not a mobile element by itself and requires chelators to maintain solvation within the plant to prevent toxicity due to the Fenton reaction. This role is fulfilled by NA which chelates several divalent transition elements such as Zn, Mn, Fe, and Ni and translocates them throughout the plant, thus partitioning the metal between source and sink (Waters and Sankaran 2011). Thus, overexpression of *NAS* has led to increased iron content in seeds, grains and aerial parts of *Arabidopsis*, tobacco, rice, and wheat (Douchkov et al. 2005; Masuda et al. 2009; Wirth et al. 2009; Johnson et al. 2011; Lee et al. 2012). Similarly, overexpression of *Malus domesticus NAS1* in tobacco significantly

increased Fe, Cu, Zn, and Mn concentrations in the flowers and leaves (Han et al. 2018). This coincided with upregulation of the iron homeostasis genes *FRO2*, *IRT1*, *VIT*, *NRAMP1*, and *YSL* as was also observed in the case of overexpression of apple *NAS* (*MdNAS1*) in tobacco, indicating alteration of metal distribution throughout the plant.

Transgenic rice overexpressing *NAS1* isolated from barley led to an increase in iron and zinc content in the grains by 4.5-fold and 2.5-fold, respectively (Masuda et al. 2009). Similar results were obtained for activation-tagged nicotianamine synthase (*Os*NAS3) from rice and iron was found to be bound to NA, rather than phytate, resulting in higher bioavailability and recovery from anemia in mice (Lee et al. 2009, 2012). The use of *At*NAS, endosperm-targeted *Pv*FER and a fungal phytase, created a source-to-sink synergetic coupling which increased endosperm iron and zinc concentrations above that achieved by ferritin overexpression alone (Wirth et al. 2009). Johnson et al. (2011) tested the potential of all three rice NAS genes toward increasing rice endosperm iron and zinc and found *Os*NAS2 and *Os*NAS3 to be effective in meeting the target of 14 ug/g DW in polished rice, again in a bioavailable form. Based on their findings, Trijatmiko et al. (2016) evaluated rice lines either expressing *Os*NAS2 alone, soybean ferritin alone or both in combination, and selected a single copy insert-containing transgenic line with combination of both genes. This line met the dietary requirements of both iron and zinc in polished grains in a bioavailable manner, without yield penalty or other side-effects on grain quality. Similarly, a combination of constitutively expressed *At*NAS1, *Arabidopsis* NRAMP3 (*At*NRAMP3), and common bean ferritin (*Pv*FER) targeted to the endosperm yielded >90% of the desired target for both Fe and Zn in grains. This approach utilized the strategy of remobilization of iron stores in the vacuole for long-distance transport via NA and ultimate sequestration by endospermic ferritin to achieve such yields, without predisposing the plants to excess Cd uptake over WT plants (Wu et al. 2019). Taken together, evidence is unanimous upon the ability of NA to provide both Zn and Fe, essential micronutrients in bioavailable form for human consumption (Zheng et al. 2010a). Interestingly, the benefits of overexpressing NAS reach beyond the obvious metallomic enrichment of crops, as nicotianamine is a potent inhibitor of circulatory mammalian angiotensin-1 converting enzyme (ACE) (Kinoshita et al. 1993), thereby aiding in the control of blood pressure, without perturbing other zinc-containing enzymes (Hayashi and Kimoto 2007). Enhancing the nicotianamine content by ~4 folds in marker-free rice through overexpression of *HvNAS1* could therefore go a long way to supplement the overall health of consumers (Usuda et al. 2009).

The above reports constitute proof-of-principles studies toward enhancing the iron content in edible parts of the plant. Progress is now being made in the arena of staple crops too. The tuberous cassava crop helps the survival of marginal and subsistence farmers in times of famine owing to its drought-resilient nature. Being a staple food of the African masses, the crop has therefore received special research attention for the introduction of superior traits through genetic engineering. The crop has been widely transformed since the first transgenic cassava produced by *Agrobacterium*-mediated transformation with a *bar* gene conferring resistance to the herbicide Basta (Sarria et al. 2000). Since then, various genes responsible for enhancing nutritional

content such as beta-carotene (Welsch et al. 2010; Failla et al. 2012), protein content (Narayanan et al. 2011), vitamin B6 (Li et al. 2015), and iron (Ihemere et al. 2012; Narayanan et al. 2015) have been explored. However, amidst the ongoing efforts to manipulate the plant intrinsic ability for iron uptake, the importance of external agronomic practices cannot be overlooked as the soil nutritional, biological, and geochemical quality is often a limiting factor in plant nutrition. Edaphic parameters are influenced by biological, anthropogenic, and weather activity, vary widely across regions and can be potential hurdles to the successful implementation of a modified crop variety. It is therefore critical to understand plant behavior in soil, especially in relation to microbial activity, given the almost universal requirement for iron. This will be discussed in the following sections along with the potential for application of beneficial interactions in iron phytofortification.

2.4 Microbe-Assisted Biofortification: The Road to Be Travelled

Soil is a highly heterogeneous medium with bulk and localized variations in pH, organic and mineral matter and moisture content, much of which is a consequence of microbial weathering (Banfield et al. 1999; Reyes et al. 2007; Sradnick et al. 2018). Microorganisms and plant roots coexist in soil with several species of microflora colonizing the root zone. Their distribution and speciation is affected by the plant species, nature and amount of exudates, secondary metabolites, plant age, stress application and nutritional status (Yang and Crowley 2000; Li et al. 2016a) meaning that the rhizosphere immediately surrounding the roots is vastly different from bulk soil (Guo et al. 2015; Shi et al. 2011). Microflora such as selected members of the genera *Pseudomonas*, *Bacillus* spp. *Azospirillum*, *Glomus* and *Trichoderma* in the rhizosphere influence plant nutrition and defense activity as well as modulate growth (Altomare et al. 1999; Ahmad et al. 2008; Rodriguez et al. 2004; Calvaruso et al. 2006; Perrig et al. 2007; Yang et al. 2009; Cassan et al. 2009).

2.4.1 Iron: The Tug-of-War Between Two Kingdoms

Iron is integral to several enzymatic reactions occurring in all aerobic organisms owing to its reactivity. However, this very same propensity toward oxidation renders it insoluble under the mostly alkaline pH of soils, triggering mechanisms for its mobilization and uptake by the organisms requiring it. Unsurprisingly therefore, soil iron availability influences the root activity of members of kingdom *Plantae* and that of the associated rhizosphere moneran community (von Wirén et al. 1999; Lemanceau et al. 2009; Marschner et al. 2011). When the iron deficiency responses of barley and sorghum were compared, it was found that root-associated microbes also

compete for the phytosiderophore, thus aggravating iron deficiency chlorosis. This was especially pronounced in sorghum, which is not as efficient a secretor of phytosiderophores as is barley (von Wirén et al. 1999). In another instance, Marschner and Crowley (1998) showed that *Pseudomonas fluorescens* Pf-5 was able to withstand iron deficiency stress when associated with the rhizosphere of barley due to the ability of this organism to utilize phytosiderophores. Characterization of the rhizosphere microbiome around barley roots under iron deficient and sufficient conditions revealed distinct communities under both conditions for which iron availability could account for 20–40% of the variation (Yang and Crowley 2000). In fact, four different root zones (the actively growing young root tips, non-growing older root tips, lateral root emergence zone, and old root axes), all, displayed communities with common and non-overlapping members, suggesting a shift in community structure with iron status. The subtleties of such variations were investigated by Robin et al. (2006a, b) who used a transgenic tobacco line overexpressing ferritin known to overaccumulate iron and therefore activate iron deficiency mechanisms. They analyzed the microbial community in the rhizosphere and on the root surface (rhizoplane) over three successive cultures in the same soil. A significant difference was obtained in the microbial fingerprint between WT and transgenic grown rhizosphere soil due to iron depletion after the first culture, but this effect was lessened and finally eliminated over the successive cultures. This occurred due to the progressive secretion of organic exudates from the iron-starved roots of ferritin overexpressing lines, which possibly decreased the iron-dependence of the rhizosphere microbial community. In contrast, *Pseudomonad* communities were found to exhibit stronger differences over culture time and at the rhizoplane level, but not in the rhizosphere, revealing the specific effect of iron depletion on this group (Robin et al. 2006b). Red clover is known to secrete phenolics for iron acquisition. One of the principal components of the exudates was found to be isoflavone (Zheng et al. 2000), a potent antimicrobial agent (Flythe and Kagan 2010). Iron-starved roots of red clover secreted high amounts of phenolics into the rhizosphere than that of roots under insufficient iron (Jin et al. 2007). The 16S ribosomal DNA fingerprinting pattern revealed different types and numbers of microbial flora around the rhizosphere of red clover dependent on the varying soil iron status, which was also confirmed by the incubation of calcareous soil with red clover phenolic extracts in place of the plant itself. Under iron deficiency, temporal variations in the microbial community were observed, with an increase in the population of quick-siderophore secreting microbes among the siderophore secretors, indicating creation of iron stress conditions. Also, the roots of these plants were found to utilize Fe-siderophore much more efficiently than Fe-EDTA (Jin et al. 2010). A similar observation was made earlier where arabidopsis supplemented with Fe-pyoverdine accumulated more iron in comparison with the plants supplemented with Fe-EDTA (Vansuyt et al. 2007). *Pseudomonas* spp. isolated from the roots of Fe stressed plants secreted a siderophore pyoverdine, which exhibits higher affinity for Fe^{+3}. Thus, this siderophore helps solubilization of the insoluble ferric ion and help plants to acquire iron (Jin et al. 2010).

2.4.2 Plant Iron Acquisition: Microbes as Supporting Actors

Plants grown in an axenic environment failed to mount a robust iron deficiency response under calcareous conditions (Masalha et al. 2000; Rroço et al. 2003; Jin et al. 2006). This implies that microbial contribution to plant iron nutrition is by no means insignificant. Indeed, microbial scavenging of iron under limiting conditions also occurs through the production of small organic molecules called siderophores such as pyoverdine, pseudobactin, enterobactin, aerobactin, desferrioxamine, and several others (Meyer and Abdallah 1978; Teintze and Leong 1981; Perry and San Clemente 1979; Barona-Gómez et al. 2004). For example, the pathogen *Erwinia chrysanthemii* produces the siderophores chrysobactin and achromobactin which stimulate a reactionary induction of ferritin in *Arabidopsis*. This induction is biphasic and required for defensive iron sequestration against utilization by the pathogen (Dellagi et al. 2005). Several reports describe the production of siderophores by root-associated microbes. Apart from mediating iron uptake by the microbe, these aid in facilitating plant access to iron through ligand exchange with phytosiderophores and defense against phytopathogenic organisms due to the sequestration of iron. The PGPR *Bacillus megaterium* isolated from the rhizosphere of tea plants displayed antagonism toward the pathogens *Fomeslamaoensis* and *Sclerotium rolfsii* and promoted plant growth through siderophore and auxin production as well as stimulation of plant defense enzymes such as chitinase and pectinases (Chakraborty et al. 2006, 2015). An increase in shoot and root dry weight as well as chlorophyll content was observed in bean inoculated with siderophore producing fluorescent *Pseudomonas* strains (Omidvari et al. 2010).

Siderophores of both plant (mugineic acids) and microbe origin pseudobactin (PB) and ferrioxamine B (FOB) have been assayed for the relative ability of plants to utilize the iron complexed to them. Accordingly, both root uptake and whole plant translocation of iron were measured to differentiate genuine plant-mediated utilization of iron from that by root adherent bacteria. Iron supplied as Fe-HMA (epi-hydroxymugineic acid) was utilized efficiently by oat and maize as measured by both high uptake and translocation, whereas Fe-FOB and Fe-PB were thought to be poor sources of iron for the plant as increased uptake did not result in higher translocation rates. This study also provided evidence for the ability of microorganisms to utilize plant siderophores (Bar-Ness et al. 1992a). However, further study with a synthetic analog of FOB revealed minor utilization of this siderophore by plants, which was masked by the dominant uptake by root-associated microbes (Bar-Ness et al. 1992b). In contrast, cucumber plants were shown to efficiently utilize FOB supplied under sterile conditions at alkaline pH, indicating possible differential preference of diverse plants for this siderophore (Wang et al. 1993). Similarly, tomatoes undergoing iron deficiency stress in alkaline soil condition were rescued from chlorosis by application of partially purified raphorin, a siderophore produced by *Rhizonusarrhizus* (Shenker et al. 1992). Similarly, siderophores of the hydroxamate family from *Penicillium*, namely fusarinines and dimerum acid (breakdown products of trihydroxamates), could act as efficient sources of iron for plants and

could easily mediate iron exchange with HMA (Hördt et al. 2000). Similarly, iron as complexed with pyoverdine was utilized by wild-type *Arabidopsis thaliana* but not as efficiently by a transgenic line overexpressing ferritin, which was instead able to assimilate iron from Fe-EDTA supplied exogenously. The overexpression of ferritin generates a pseudo-iron deficient state due to overaccumulation of iron and subsequent upregulation of root IRT1, implying that the Fe-pyoverdine complex is not imported into the plant via IRT1 (Vansuyt et al. 2007). The effectiveness of microbial siderophores in augmenting plant iron reserves may also depend on the intrinsic capacity of the plant and the strain of microorganism used. For instance, *Rhizobium* species were able to enhance iron content in common bean, whereas *Pseudomonas* strains could increase both Fe and Cu content. In both cases, the wild-type bean responded more efficiently compared to the cultivated varieties (Carrillo-Castañeda et al. 2005).

2.4.3 Microbial Aided Plant Growth: Mechanisms Centering on Iron

The stimulatory effect of plant growth promoting rhizobacteria (PGPRs) is well documented (Berg 2009). Microbes can promote plant growth through two major mechanisms: (1) directly such as by the production of plant hormones (auxins, ethylene, cytokinins, gibberellins, and abscisic acid), nitrogen fixation and increasing accessibility to soil nutrients and (2) indirectly by antagonism against pathogens (antibiosis, production of VOCs, cyanide, membrane denaturants) and stepping up plant defense response (called induced systemic resistance ISR) (Ali et al. 2009). Plant fitness is also improved through the protective function of PGPRs against heavy metal toxicity by restricting uptake and secreting plant hormones for sustained growth under stress. The plant hormone abscisic acid (ABA) when externally supplied at root level in soil was shown to block IRT1-mediated uptake of cadmium (Cd) in *Populus euphratica*, along with enhancing enzyme activities for scavenging stress-induced free radicals (Fan et al. 2014; Han et al. 2016), thus sustaining plant growth in the presence of Cd. Though this phenomenon offers great potential for the remediation of contaminated soil, the hormone itself is poorly stable in soil conditions (Xu et al. 2018). A number of PGPRs, however, produce ABA (Perrig et al. 2007, Boiero et al. 2007). For instance, inoculation of *Bacillus subtilis* or *Azospirillum brasilense* in cadmium-contaminated soil reduced the accumulation of cadmium in the plant compared with the levels in non-inoculated soil (Xu et al. 2018). Dead bacterial culture failed to protect the plant from Cd toxicity, while the addition of live culture could rescue the plants from Cd stress. It was found that the secretion of abscisic acid (ABA) by these bacteria suppressed the expression of the poorly selective root transporter IRT1 (Korshunova et al. 1999), thereby affording similar protection to the plant as with exogenous ABA.

Interestingly, enhancements in plant growth and seed set were also observed in the case of PGPRs of *Bacillus* spp. such as *B. subtilis* GB03 even when they are not in physical contact with the plant roots (Farag et al. 2006; Zhang et al. 2008; Xie et al. 2009). For example, *Arabidopsis* was cultured in Petri plates with a physical barrier separating a PGPR *Bacillus amyloliquefaciens* colony and the plants, on sterile agar medium, such that there was no physical contact between the two organisms (Wang et al. 2017). These bacteria are known to emit volatile organic compounds (VOCs) such as 2, 3-butanediol, acetoin, methyl-3-butanal, 2-methyl-1-propanol, and 3-methyl-1-butanol, which promote plant growth through various mechanisms such as inducing systemic resistance against pathogens, auxin signaling for root growth and inhibition of sugar mediated feedback repression of photosynthesis (Ryu et al. 2004; Farag et al. 2006; Zhang et al. 2007, 2008; Gutiérrez-Luna et al. 2010). These VOCs also induced the strategy I plant iron acquisition mechanism involving FIT1-mediated induction of *FRO2* and *IRT1* (Zhang et al. 2009). This phenomenon closely parallels the observations noted upon the inoculation of Chinese milkweed plants with the PGPR *Burkholderia cepacia* (Zhou et al. 2018). This bacterium significantly improved photosynthesis, maintained chloroplast structure, and enhanced rhizosphere acidification in calcareous soil, compared with un-inoculated plants. Strategy I responses such as IRT1, FRO2, and AHA pump were elevated and enhanced the secretion of riboflavin and its derivatives were observed in inoculated plants compared to control, even under non-calcareous soils. These mechanisms, likely mediated in part by IAA secretion from *B. cepacia*, allowed inoculated plants to accumulate significantly more iron compared to un-inoculated controls under hostile soil conditions.

Plants exposed to VOCs were more tolerant to calcareous conditions due to VOC-induced NO production, which promoted utilization of apoplast-precipitated iron and translocation within the plant body. VOC-exposed plants were able to photosynthesize better than their unexposed counterparts through increase in chlorophyll content, photosystem II efficiency (Zhang et al. 2008; Xie et al. 2009) and stabilization of chloroplast structure under Fe deficiency, due to the efficient utilization of iron. Additionally in case of *B. subtilis* GB03, the VOCs emitted could transiently (over 3 days) induce transcripts of cell wall expansin 5 (*EXP5*), and *IRT1* in the exposed arabidopsis plants, leading to growth due to loosening of cell walls and iron uptake to meet growth requirements. However, such enhancement in plant growth required long periods of exposure to VOCs, with only airborne interaction between plant and microbe, thus establishing the essential role of microbial VOCs in plant signaling and behavior (Xie et al. 2009).

Iron is at the heart of plant defense responses as it shifts to the apoplast upon pathogen attack with subsequent H_2O_2 production and upregulation of pathogenesis-related genes, all of which form a sustained amplified loop (Liu et al. 2007). The secretion of plant phytosiderophores and microbial siderophores is also well known as discussed earlier, prompting the suggestion that the two may interact either competitively or beneficially to facilitate iron uptake by the plant. One of the mechanisms by which PGPRs were proposed to act was through the selective sequestration of iron by efficient siderophores, thus making already scarce soil iron further unavailable to

competitors such as pathogens (Kloepper et al. 1980). Plant growth promoting rhizobacteria was long known to both enhance plant iron nutrition and prime the plant defenses against pathogen attack (called induced systemic resistance ISR) through siderophore-mediated competitive sequestration of iron (Press et al. 2001; Segarra et al. 2010). In roots of arabidopsis associated with *P. fluorescens* WCS417, 135 genes common to both iron deficiency response and PGPR inoculation were upregulated, as were marker genes of iron deficiency, namely MYB72, FRO2, and IRT1 (Zamioudis et al. 2015), hinting at commonalities between the iron deficiency response and PGPR action. Herein, the iron deficiency responsive TF MYB72 was shown to play a pivotal role in coordinating both activities. This TF facilitates the strategy I response under iron deficiency by inducing root NAS4 (Palmer et al. 2013) as well as secretion of iron-solubilizing phenolics into the rhizosphere through the action of BGLU42, a β-glucosidase (Zamioudis et al. 2014). Very recently, it was shown that ISR induction in arabidopsis by the PGPR *Pseudomonas simiae* WCS417 was dependent on MYB72 regulated production and secretion of the plant phenolic scopoletin which has strong antimicrobial activity against soil-borne fungal pathogens but sustains symbiosis between the PGPR and host (Stringlis et al. 2018). Furthermore, only ISR-inducing PGPRs (*Pseudomonas* spp, *Actinobacteria* spp, *Trichoderma* spp) essentially activate the FIT-dependent typical iron deficiency response in arabidopsis roots by producing VOCs which upregulate MYB72coexpressing with FRO2 and IRT1 (Van der Ent et al. 2008; Segarra et al. 2009; Zamioudis et al. 2015). Interestingly, this response was independent of the external iron concentration in the soil, suggesting interference with the iron status and sensing in the plant. Indeed, exposure of roots to VOCs repressed the iron status marker ferritin (*FER1*) in the roots, indicating the depletion of cellular iron reserves through possible increase in metabolic activity of root cells requiring iron. However, VOC-mediated MYB72 activation was fully dependent on a shoot-derived photosynthetic signal, which communicates the iron status of the plant to the late maturation zone (LMZ) of the root. Accordingly, VOC-mediated induction of MYB72 and strategy I response occurred only in the LMZ of arabidopsis roots and was required to augment the standalone root-autonomous response to iron deficiency which occurs in the early maturation zone (EMZ) and apex, for optimal whole root iron deficiency response. In addition, nitric oxide (NO) activity was found to be essential for VOC-mediated activation as MYB72 induction was abolished in plants treated with an NO scavenger (Zamioudis et al. 2015). With respect to the role of PGPR VOCs in ISR (such as that induced by *Trichoderma* spp), recent work showed that the MYB72-directed targeted alterations in root architecture were concomitant with the induction of the jasmonic acid-defense responses in the upper parts of the plant (Martínez-Medina et al. 2017). Taken together, the ability of root-associated rhizobia to sense and manipulate the host plant iron status facilitates enhanced plant iron acquisition from the rhizosphere, while simultaneously conferring ISR for improved fitness (Fig. 2.1).

Endophytic PGPRs are also able to exert protective effects on their host plants. The detrimental effects of 5 mM iron (toxic) on wheat seedlings were ameliorated when they were incubated with the endophyte *B. altitudinus* WR10. This organism was found to synthesize the auxin indole acetic acid (IAA), thereby relieving inhibition

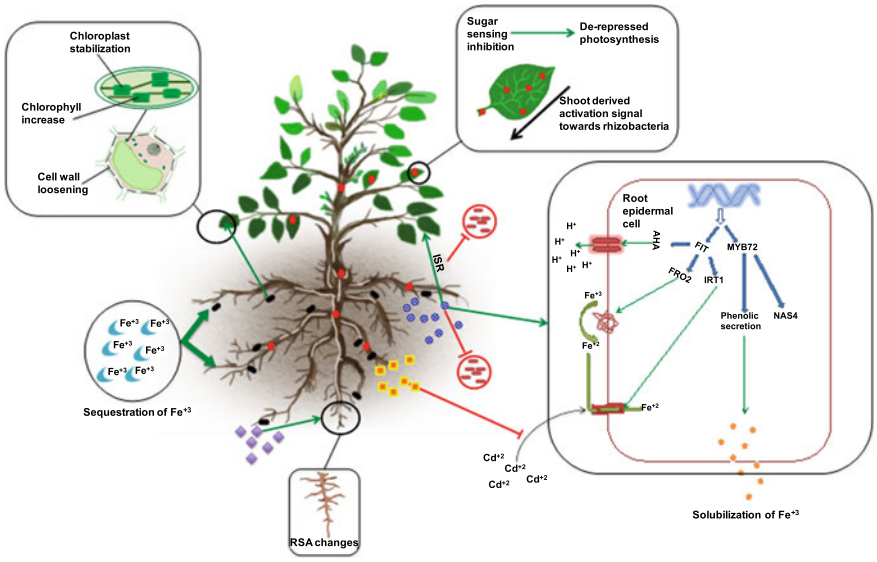

Fig. 2.1 Plant–microbe interactions centering on iron. Plant root adherent rhizobacteria enhance plant growth by influencing plant systemic resistance (ISR) against both root and foliar pathogens, enhancing photosynthetic capacity through chloroplast stabilization, sequestering iron (Fe^{+3}) with siderophores, inducing the iron deficiency response through FIT and MYB72, and finally by releasing volatile organic compounds (VOCs). **Key**: Foliar/Root pathogens, Rhizobacteria, sugars, Plant phenolics, Bacterial VOCs, Bacterial IAA, Bacterial ABA, Microbial siderophores, Activation, Inhibition, FRO2, AHA, IRT1, used by both rhizobacteria and plant roots

of root elongation under iron toxicity. Furthermore, under iron toxicity and in the presence of WR10, wheat sprouts upregulated ferritin genes in their roots while downregulating them in the shoots. This prevented excess iron from being translocated to the sensitive shoots, which perform active photosynthesis. The downregulation of the transporters NAM, ZIP, and UPP in both shoot and root also contributed to the prevention of iron toxicity (Sun et al. 2017).

2.4.4 Microbes in Field: Potential for Application

Application of iron salts to the soil alone is not recommended as iron tends to precipitate and become unavailable. Currently, standard agronomic interventions toward iron enrichment of crops aim to lower the pH of calcareous soils to facilitate iron uptake by plants. Accordingly therefore, methods such as foliar application of iron salts along with nitrogen (Aciksoz et al. 2011), the use of a mix of organic and inorganic fertilizers to lower soil pH and increase organic acid-based solvation of iron (Ramzani et al. 2016) as well as intercropping with graminaceous plants which

release phytosiderophores (Xiong et al. 2013) are some of the methods utilized. Attention is now increasingly being focused upon the use of rhizosphere-associated microbes as natural means to increase iron content in edible parts of the plants as they provide a host of other benefits to the plant as detailed in the preceding paragraphs.

When considering microbial assisted biofortification, the soil pH and other parameters play an important role in determining the efficacy of the above-mentioned phenomenon. For instance, though arbuscular mycorrhizal (AM) fungi are known to enhance nutrient uptake by plants (Mohammad et al. 2003), soil pH increase drastically reduced the ability of the AM fungus *Glomus versiforme* to colonize roots of the iron deficiency susceptible *Citrus* species *Poncirus trifoliate* (Wang et al. 2008). However at pH 6 (optimal for this species of *Citrus*), this fungus enhanced chlorophyll content, shoot biomass and iron content in *Poncirus trifoliata* compared to un-inoculated controls, by increasing root ferric chelate reductase activity. Thus AM-inoculated plants had better iron status than un-inoculated plants as shown by lower P/Fe and 50(10P+K)/Fe ratios which are markers for iron chlorosis, indicating better acquisition by this iron deficiency susceptible plant (Wang et al. 2008).

Increased growth and yield in chickpea was observed when inoculated with PGPR and iron. The PGPR helps in nitrogen fixation, phosphate solubilization along with the production of phytohormones (IAA) and organic acids, which promote plant growth. PGPR-treated plants also showed increased iron content in their root, shoot, and grains (Khalid et al. 2015). The microbiota associated with the roots not only increase iron uptake by plants and plant growth but also act as effective soil conditioners through varied integrated mechanisms described earlier. They thus prove superior to conventional inorganic additives and pesticides with deleterious environmental impact and can be thought of as a wholesome tool to cater to the nutrient and growth needs of the plant. Overall, the involvement of microbes in iron acquisition seems to be a promising tool toward iron phytofortification with the far-reaching aim of alleviating iron deficiency anemia.

2.5 Conclusion and Perspective

Taken together, an insight on iron homeostasis pathway and ways to increase iron content by the associated microbes can help in increasing the nutritional quality of the agricultural produce. Why consider microbes in the arsenal against plant iron deficiency? They can improve stress tolerance in the plant, availability of other nutrients such as P, potentially leading to phasing out of unsustainable inorganic NPK fertilizers which cause eutrophication and also afford protection against a variety of plant pathogens. Thus, the overall quality of the soil with respect to plant nutrition is improved, in a cost-effective manner. Integrated management of agricultural practices coupling modern technologies with agronomy holds the potential to facilitate the generation of quality food for the ever-burgeoning populace with shrinking natural resources. In this regard, the rhizosphere microbiota will fast become an indispensable tool toward iron phytofortification.

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Chapter 3
Paradigm Shift from Marker-Assisted Breeding to Genomics-Assisted Breeding for Calcium Nutrition in Finger Millet

Divya Sharma, Salej Sood and Anil Kumar

Abstract The rapid advancements in molecular marker technologies followed by genomics, and next-generation sequencing technologies in major crops like rice, maize, and wheat have given opportunities for their use in the minor, but highly valuable future crops, including finger millet (*Eleusine coracana* (L.) Gaertn.). Finger millet, an orphan crop has an immense potential as a nutritional security crop due to its exceptionally high calcium content. Calcium (Ca), considered to be the most essential macronutrient, is required in relatively large quantities in the diet for maintaining healthy state of body. The unavailability of sufficient markers and genome sequence information in finger millet has resulted in limited breeding efforts for nutritional quality improvement through marker-assisted breeding. Nonetheless, advances in large-scale genomics technology have now streamlined production of genome-wide markers which can be used for large-scale identification of candidate genomic loci. The availability of NGS-based approaches with high resolution has enhanced the pace, precision, and efficiency of trait mapping. At present, trait-associated markers, cost-effective genotyping platforms and expertise are available for deploying genomics-assisted breeding in finger millet. High-throughput genotyping technology and phenotyping platforms have enabled genome-wide association studies, to precisely dissect the genetic architecture of complex traits such as calcium nutrition in finger millet. Large-scale mapping of agronomically important quantitative trait loci would not only help in the identification of molecular markers linked to grain calcium content in finger millet but also help in gene cloning and characterization, mining of elite alleles, exploitation of natural variations, and genomic selection paving the way toward genomics-assisted breeding, which ultimately will lead to genetic

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enhancement of finger millet. Therefore, marker-assisted breeding and selection is gradually evolving into 'genomics-assisted breeding' for crop improvement.

Keywords Malnutrition · Calcium · Biofortification · Bioavailability · Finger millet · Marker-assisted breeding · Speed breeding · Genomics

3.1 Introduction

Mineral malnutrition is affecting one half of the world's population. People have no access to a variety of minerals such as iron, calcium, zinc, magnesium, and copper. It is estimated that as many as three billion people may suffer from mineral deficiency (Graham et al. 2001). The vast majority lives in poverty and lack access to a secure supply of safe and nutritious food, meaning they achieve neither an adequate food intake nor the reference daily intakes (RDIs) of mineral nutrients for healthy individuals. Most of the staple food crops such as rice, wheat, and maize which constitute the major part of the diet of people are often deficient in these macro/micronutrients, thus insufficient to meet the daily needs (Hirschi 2009). Deficiency of these minerals leads to an increasing risk of diseases such as rickets, osteoporosis, anemia, and hypertension. It has been estimated by the Food and Agriculture Organization (FAO) that the world's population will reach 9.1 billion by 2050 (Food and Agriculture Organization (FAO) of the United Nations 2009). Hence, to meet the food demands of such a large population, the quantity as well as the quality of food needs to be improved in terms of their nutritional value.

3.1.1 Mineral Deficiencies: The Case of Calcium

Calcium is the fifth most abundant element present in the human body, accounting for up to 1.9% of the body weight in adults (Nordin 1976). Its main functions are to provide rigidity and structure, mediating vascular and muscular contractions or dilations and nerve signal transmission (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes 1997; Nordin 1997). Ca may also serve in the protective role against various types of cancer, viz., colorectal (Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium 2011), ovarian (Goodman et al. 2002), breast (Lin et al. 2007), and prostate (Gao et al. 2005). Although not supported by clinical trials, observational studies have associated higher Ca intakes to lower body weight and reduced adiposity, which may be due to lower intracellular Ca in fat cells leading to a higher fat breakdown (Parikh and Yanovski 2003). Thus, it may reduce the risk of cardiovascular diseases by lowering intestinal lipid absorption, promoting lipid excretion, and decreasing cholesterol concentrations in the blood. Given its importance, authorities like the Food and Agriculture Organization (FAO) of the United Nations have set

up a recommended daily intake (RDI) of Ca based on age, life stage, and gender (Food Agricultural Organization of the United Nations 2002). During the phases of active growth, Ca equilibrium in the body maintains a stable bone mass. Therefore, FAO recommends that children of 1–3 years consume 500 mg Ca/day, 4–6 years consume 600 mg Ca/day, and 7–9 years consume 700 mg Ca/day, which should be increased to 1300 mg/day during 10–18 years (Food Agricultural Organization of the United Nations 2002). About 1000 mg Ca/day is recommended between the ages of 19–65 years in males. The organization also advocates that women should take 1000 mg Ca each day from 19 years onward until menopause raising it to 1,200 mg during the last trimester of pregnancy, and to 1300 mg from 65 years and above (Food Agricultural Organization of the United Nations 2002). Despite the importance of adequate Ca intake for human health and wellbeing, the WHO estimates that low dietary intake of Ca is common across the world (Villar et al. 2006). Most of these regions have an agriculture-based economy on what they grow and produce for their Ca need. In such situations, staple crops that can offer adequate Ca requirements, especially for people of low-income groups in these countries, are highly recommended. One such Ca-rich, traditionally and locally well-adapted crop is finger millet. As opposed to nutritionally deficient cereals, such as rice, its regular consumption has a vast potential to curb the incidences of Ca deficiency.

3.2 Biofortification: Combating Mineral Malnutrition

Biofortification is the development of nutrient-dense staple crops using the best conventional breeding practices and modern biotechnology, without sacrificing agronomic performance and important consumer-preferred traits (Nestel et al. 2006). Plants are the ultimate sources of nutrients in human diet. However, majority of the essential vitamins and minerals are lacking in all our staple food crops. Although, a balanced diet provides sufficient nutrients but most of the human population, particularly in developing countries depends upon staple cereals, such as rice or maize, which fail to provide the full complement of essential nutrients. Malnutrition has become a significant public health issue in most of the developing world (Müller and Krawinkel 2005). One way to tackle this problem is through the enrichment of staple crops to increase their essential nutrient content. Several different tactics for biofortification have been adopted including addition of the appropriate mineral as an organic compound to the fertilizer, improving the nutritional content of plants by conventional breeding in combination with mutagenesis and the use of marker-assisted selection to introgress such traits into widely cultivated, adapted genotypes. Although breeding-based strategies for biofortification are unproven as yet, they have the potential to become sustainable, cost-effective and reach remote rural populations (Bouis 2003; Genc et al. 2005). It is argued that once mineral-dense lines have been developed, there will be little additional cost in incorporating them into ongoing breeding programs (Welch and Graham 2002; Bouis 2003; Timmer 2003), and it has been reported that seed of mineral-dense crops produces more vigorous seedlings on

infertile soils (Rengel and Graham 1995). To implement successful biofortification programs through plant breeding, there is a need for a comprehensive exploration of potential genetic resources in the form of land races, wild species, and an in-depth understanding of the physiological and genetic basis of mineral nutrients accumulation in staple food crop. It therefore becomes necessary to understand the genes and processes involved in grain mineral accumulation in order to couple the information with marker/genomics-assisted selection, for efficient enhancement of grain mineral content. A breeding program aiming at development of new genotypes with high Ca^{2+} concentration first requires the existence of useful genetic variation for Ca^{2+} accumulation in grain. Little information is, however, available about the genetic control and molecular physiological mechanisms contributing to high accumulation of Ca^{2+} and other micronutrients in grain of different genetic materials (Sharma et al. 2017).

3.3 Bioavailability: A Complex Determining Factor to Achieve Adequate Intake of Calcium from Biofortified Finger Millet

Determining the efficacy and biological impact of Ca-biofortified finger millet and other cereals on better nutrition and improved health is very challenging. It depends mainly on two processes: bioaccessibility and bioavailability of Ca in the seeds. Bioaccessibility is a measure of the nutrient fraction available for absorption after its release from food matrix in the gastrointestinal tract. On the other hand, bioavailability is a utilization-based definition, where the ingested, digested, and absorbed nutrient reaches the systemic circulation and exerts a positive effect on health (Carbonell-Capella et al. 2014). Plant foods contain substances (i.e., anti-nutrients) that interfere with the absorption or utilization of these nutrients in humans (Welch and Graham 1999). Thus, efforts should be made toward increasing the concentrations of "promoter substances" (stimulating the absorption of essential mineral elements) and reducing the concentrations of "anti-nutrients" (interfering with their absorption) of the biofortified crops (White and Broadley 2005). Finger millet contains both water-soluble and liposoluble vitamins: thiamin, riboflavin, niacin, and tocopherols (Obilana and Manyasa 2002), which could act as potential promoter substances for crop biofortification. Utilization of the maximum nutrient potential of the millets is limited by the presence of phytates, phenols, tannins, and enzyme inhibitors. Among millets, finger millet has been reported to contain high amounts of tannins ranging from 0.04 to 3.74% of catechin equivalents (Rao et al. 1994; Antony and Chandra 1999). Phytate content in finger millet as observed by various authors has been found to be in range 0.679–0.693 g/100 mg (Antony and Chandra 1999). It is the main phosphorous store in mature seeds which has a strong binding capacity and readily forms complexes with multivalent cations and proteins (Haug and Lantzsch 1983). Finger millet has been found to contain 41% phytic phosphorus

as percentage of total phosphorus (Deosthale 2002). The dietary phytic acid binds not only with the seed-derived minerals but also with other endogenous minerals encountered in the digestive tract (Raboy 2000). Another group of anti-nutritional compound is polyphenols, which contains more than one phenol unit or building block per molecule (Carvalho and Vasconcelos 2013). The level of polyphenols in cereal seeds can be reduced by incubation with polyphenol oxidase which, when combined with a phytase-mediated phytate reduction, shows a significant increase in the availability of iron (Matuschek et al. 2001). On an average, finger-millet genotypes contain 0.04–3.47% polyphenols (Chethan and Malleshi 2007). Rao and Muralikrishna (2002) found proto-catechuic acid (45.0 mg/100 g) as the major free phenolic acid in finger millet grains. Among bound phenolic acids, ferulic and *p*-coumaric acids are the major fractions and account for 64–96 and 50–99% of total ferulic and *p*-coumaric acid content of finger millet grains, respectively (Devi et al. 2014). Numerous complexities pervade the determination of bioavailability of micronutrients in plant foods to humans. Determining the bioavailability of a particular micronutrient to an individual eating, a mixed diet in a given environment is actually governed by the interaction of a multitude of factors (Fairweather-Tait and Hurrell 1996; House 1999; Van Campen and Glahn 1999; Graham et al. 2001). Through plant-breeding approaches, one could select genotypes with low concentration of anti-nutrients or alternatively, molecular biologists alter genes in staple crops so as to reduce or completely eliminate these anti-nutrients. For enhanced Ca bioavailability from finger millet, grain Ca content needs to be improved with a concomitant but conscious effort for the reduction of anti-nutrient compounds. This is because these compounds play a vital role in plant development and survival. For example, finger millet tannins are effective in reducing pre- and post-harvest losses as they provide protection against molds, insects, and other abiotic stress (Gull et al. 2014). Similarly, phytic acid acts as the main phosphorus store for the seeds (Singh and Raghuvanshi 2012). These compounds have also called attention due to their nutraceutical value and protective effects against many chronic diseases (Kumar et al. 2016a). Thus, their importance can never be completely disregarded. Engineering their content to become a non-limiting factor in Ca absorption from finger millet must be done in a way that does not negatively affect crop performance. A justified way to accomplish this is by employing efficient and suitable grain-processing techniques.

3.3.1 Host Factors Influencing Calcium Bioavailability

Apart from the Ca bioavailability parameters, the capability to determine the effect on Ca status on target populations is another specific challenge. Host factors, such as age, gender, and dietary patterns may show differential effects of finger-millet-based diets on the net Ca contribution. These factors must be considered in controlled feeding community-based studies to determine the biological impact of biofortified crops. In the past, various attempts have been put together to assess the Ca bioavailability in vivo. Early nutrition studies have shown that rats fed with a diet composed of

70% finger millet retain 68% Ca. A further reduction of the finger millet content to 20–40% in diets contributed to increased Ca retention to 84–88% levels (Giri 1940). This shows that even a low-dietary component of finger millet is sufficient to maintain Ca availability because of its high Ca content. In fact, in a more recent study, Ca from finger millet had shown to have a better uptake as compared to commercial Ca supplementation tablet (Bhide et al. 2013). However, for human metabolism studies, host factors like age and gender are important parameters to estimate daily requirement, intake, and retention of dietary Ca. Many nutrition reports have estimated the contribution of finger millet for Ca homeostasis in humans. A study by Subrahmanyan et al. (1955) had found that a finger-millet variety, H22, with Ca content 440 mg/100 g can on an average provide 3.4 g Ca/day to healthy adult males aged 22–32 years. This amounted to Ca retention of 98 mg (approximately 3%) from a total daily intake of 3.4 g/day. This was higher than a brown bread or Ca carbonate fortified brown bread diet providing only 0.5–1.2 g Ca/day, respectively. It is recommended that diets should provide at least 200 mg/100 g of Ca to counteract the anti-calcifying effect of phytic acid (McCance and Widdowson 1942). Interestingly, 86% of phytate ingested from the finger-millet-based diet was hydrolysed during digestion and absorption process (Subrahmanyan et al. 1955). As most of the phytate is broken down during digestion, therefore, regular inclusion of finger millet in diet can efficiently maintain a positive Ca balance. Such information can allow the acceleration of finger-millet biofortification programs.

3.4 Finger Millet: A Model Crop for Calcium Biofortification

Finger millet is a potential staple crop cultivated mostly in Eastern and Central Africa and India. It ranks fourth in importance among millets in the world after sorghum, pearl millet, foxtail millet and commonly referred as ragi, mandua bird's foot millet, coracana, and African millet (Upadhyaya et al. 2007). Nutritionally, finger millet is an excellent source of nutrients, especially calcium, other minerals, and dietary fiber. The mineral composition of finger millet grains is highly variable. The mineral content of these food grains is affected by the presence of genetic factors and environmental conditions prevailing in particular growing region (Singh and Raghuvanshi 2012; Singh et al. 2014). Finger millet contains a fair amount of protein (7.3%) (Malleshi and Klopfenstein 1998), dietary fiber (15–20%) (Chethan and Malleshi 2007), and a rich source of calcium (Ca^{2+}) (344 mg 100 g^{-1}) (Gopalan et al. 1999) and iron (3.7–6.8 mg/100 g) (Barbeau and Hilu 1993). Ca^{2+} analysis of 36 genotypes of finger millet was carried out; concentration varies from 162 to 487 mg/100 g grain with mean value of 320.8 mg/100 g grain (Vadivoo et al. 1998). The average Ca^{2+} content (329 mg/100 g grain) in white varieties was considerably higher than the brown (296 mg/100 g grain) varieties (Seetharam 2001). Bhatt et al. (2003) reported the Ca^{2+} content of finger millet as 344 mg/100 g grain, which is 5–30 times more than most cereals (US NRC 1996). High grain Ca^{2+} content as

high as 450 mg/100 g grains has been reported in finger millet (Panwar et al. 2010). Babu et al. (1987) reported that among six hybrid varieties of finger millet, calcium varies from 293 to 390 mg/100 g. Ravindran (1991) estimated the protein content of ragi to be 9.8%, that of calcium, oxalate, and phytic acid to be 0.24, 0.44, and 0.48%, respectively. Admassu et al. (2009) measured the proximate composition of six varieties of finger millet. The values ranged from 50 to more than 300 mg/100 g for calcium content. In the ICRISAT core collection of finger millet, the calcium content up to 489 mg/100 g was recorded in the genotype IE 4476 (Upadhyaya et al. 2011b). The yield potential for finger millet is in the range of 4–5 tons/ha (FAO 2008). Thus, finger millet is upcoming as an important food crop due to its exceptionally high calcium content. It is not only an excellent source of dietary calcium but also an excellent model to explore the genetic control and molecular mechanisms contributing to high grain Ca^{2+} content (Sharma et al. 2017) (Table 3.1).

3.4.1 Exploiting Existing Genetic Variation: Prerequisite for Biofortification

Natural variation embraces the enormous diversity present within wild plant species as well as most of the genetic variants that are found in domesticated plants. There is a substantial natural variation for mineral use efficiency, root uptake, translocation from roots to shoots, and accumulation in the seed as storage and supply for the germinating seedling. This variation has been reported in many species, leading to breeding programs such as those aiming to improve zinc and iron status of cereal grains or tuber crops (www.harvestplus.org). However, most of the natural variation is quantitative and determined by molecular polymorphisms at multiple loci and genes (multigenic), which are referred to as quantitative trait loci (QTL) and quantitative trait genes (QTGs). The natural variation present in crop plants has been exploited since their domestication thousands of years ago by the genetic manipulation of developmental traits and physiological features related to adaptation to agriculture.

In recent years, various efforts have been made by geneticists and breeders to identify naturally occurring genetic diversity in finger millet. However, the major challenge at present is how these resources could be exploited to develop Ca-biofortified finger millet (Puranik et al. 2017). Currently, finger-millet gene banks across the globe conserve more than 37,000 accessions with India, Kenya, Ethiopia, Uganda, and Zambia, housing the major collections. As of now, the entire genetic diversity present among the finger millet germplasm is available as small sets (core) and subsets (mini core) collections (Vetriventhan et al. 2015). Using these collections, 15 accessions were identified as most promising (3.86–4.89 g/kg) for further improving grain Ca content in cultivated finger millet (Upadhyaya et al. 2011b). Recently, another core set of 77 germplasm of Indian and African origin has been formed using the base germplasm of finger millet 1000 accessions (Chandrashekhar et al. 2012).

Table 3.1 Calcium content of millets and cereals (per 100 g)

Crop	Calcium content (mg/100 gm edible portion)	References
Millets		
Finger millet (*Eleusine coracana*)	344	Shobana et al. (2013)
Teft (*Eragrostis teff*)	78.8–147	Baye (2014)
Fonio (*Digitaria exilis*)	44	National Research Council (1996)
Pearl millet (*Pennisetum glaucum*)	42	Shobana et al. (2013)
Foxtail millet (*Setaria italica*)	31	Shobana et al. (2013)
Kodo millet (*Paspalum scrobiculatum*)	27	Shobana et al. (2013)
Barnyard millet (*Echinochloa crus-galli*)	20	Shobana et al. (2013)
Little millet (*Panicum sumatrense*)	17	Shobana et al. (2013)
Proso millet (*Panicum miliaceum*)	14	Shobana et al. (2013)
Common cereals		
Wheat (*Triticum aestivum*)	41	Shobana et al. (2013)
Rice brown (*Oryza sativa*)	33	Saleh et al. (2013)
Corn (*Zea mays*)	26	Saleh et al. (2013)
Sorghum (*Sorghum bicolor*)	25	Saleh et al. (2013)
Barley (*Hordeum vulgare*), raw	20	McKevith (2004)
Rye (*Secale cereale*), Flour	20	McKevith (2004)
Oatmeal (*Avena sativa*)	52	McKevith (2004)
Rice (*Oryza sativa*) raw Millet	10	Shobana et al. (2013)

In addition, finger-millet composite collections (1000 accessions) and a derived reference set (300 accessions) representing region and race-based available diversity of the entire collection, is also available (Upadhyaya et al. 2005; Upadhyaya 2008). GPHCB-45, a variety of finger millet, has been registered as high-calcium variety of finger millet with 452.8 mg/100 g calcium content (National identity, IC0614156). Although these large collections of finger millet germplasm serve as an ideal resource to be utilized in improving its Ca concentration, a majority of it remains largely underutilized for breeding high Ca finger millet varieties. Some of the main reasons for this lag are due to factors, such as weak and insufficient strategies for harnessing the useful genetic diversity available in these collections, barriers related to introduction and crossing of exotic germplasm, few pre-breeding programs to facilitate

introgression of desirable nutrition quality into breeding lines and recirculation of same working collections by breeders (Dwivedi et al. 2009; Upadhyaya et al. 2014). At the same time, just selecting suitable donor lines for selective breeding based on variation in grain Ca content is not sufficient and may not even be successful as such variations may often be regulated at various other levels. Therefore, determination of genetic stability and adaptability of this trait across multiple environments is one of the prerequisite to develop effective strategies for breeding elite lines (Puranik et al. 2017) (Table 3.2).

3.4.2 Precise Phenotyping of Complex Traits like Calcium Nutrition

However, little is known about the genes, their location, their effect on a particular trait, and their interactions with each other (epistasis) (Kearsey and Farquhar 1998). With the tremendous advancements in the area of genomics in the recent past, the situation has changed a lot. We can now predict the location, function, nature, and interactions of a gene with maximum certainty. However, the precision of prediction of genes and their function through genomics needs to be enhanced by making use of high-throughput phenomics tools and technologies. This will help us to harness the full benefits of genomics for crop improvement programs. Meaningful QTL/gene discovery programs through QTL/association mapping require precise and accurate phenotyping data of complex traits. It is important to mention here that valid and accurate results reported with non-conventional techniques so far have not yielded expected results, in spite of huge molecular genotypic data generated during the past few years (Edmeades et al. 2004; Araus et al. 2008; Xu and Crouch 2008). One important reason is the slow progress in the area of phenomics which involves a number of approaches for recording precise high-throughput phenotypic data. Phenotypic data is the primary data required for the genetic dissection of complex phenotypic traits and hence should be taken precisely. To obtain a clean set of precise and reproducible phenotypic data of complex quantitative traits like calcium nutrition from large germplasm collections still remains a challenge today even in the era of phenomics driven technology. Phenotypic data should be taken with care since such data is highly influenced by environmental variations and are thus more prone to experimental error. Therefore, the gap between genotype and phenotype can only be filled with proper, accurate, and precise phenotyping of quantitative traits (Tuberosa 2012). The precision with which the chromosomal regions are identified and their effects are accurately estimated depend upon how precisely the phenotypic data are recorded to establish the phenotype–genotype association. In other words, the use of molecular approaches for crop improvement depends upon how well and how accurately the target trait has been assessed phenotypically in mapping population or diversity panel because if the phenotypic data is not taken accurately, there will be more chances of false positives and false negatives. Good phenotyping increases accuracy, precision,

Table 3.2 Status of millets germplasm accessions conserved in ICRISAT and ICAR gene bank

Crop	Total Number of accessions	Number of countries	Number of races	Number of traits used for constituting core and minicore collection	Number of accessions in core collection	Number of accessions in minicore collection	Number of accessions in GCGC collection	References
ICRISAT germplasm collection								
Sorghum	39,234	93	15	21	2247	242	3367	Grenier et al. (2001), Upadhyaya et al. (2009, 2015)
Pearl millet	22,658	50	–	22 for core and 12 for minicore	2094	238	1021	Upadhyaya et al. (2010, 2011a)
Finger millet	6084	24	5	14 for core and 18 for minicore	622	80	959	Upadhyaya et al. (2009, 2011b)
Barnyard millet	749	10		21	89	–	–	Upadhyaya et al. (2014)
Proso millet	849	20		20	106	–	–	Upadhyaya et al. (2011b)
Foxtail millet	1542	26		23 for core and 21 for minicore	155	35	452	Upadhyaya et al. (2011c)

(continued)

Table 3.2 (continued)

Crop	Total Number of accessions	Number of countries	Number of races	Number of traits used for constituting core and minicore collection	Number of accessions in core collection	Number of accessions in minicore collection	Number of accessions in GCGC collection	References
Kodo millet	665	2		20	75	–		Upadhyaya et al. (2014)
Little millet	473	5		20	56	–		Upadhyaya et al. (2014)
NBPGR germplasm collection								
Sorghum	20,376	–	–	–	–	–		Hariprasanna et al. (2015)
Pearl millet	7841	–	–	–	–	–		Hariprasanna et al. (2015)
Minor millets	23,864	–	–	–	–	–		Hariprasanna et al. (2015)

and throughput at all levels of biological organization while reducing cost and minimizing labor through automation, data integration, and experimental design (Cobb et al. 2013).

3.4.3 Challenges Associated with Breeding-based Genetic Improvement of Finger Millet

As finger millet is a naturally self-pollinating crop, artificial hybridisation by crossing of suitable parental lines is often a difficult task. Mass and pure-line selection practices have come in handy for inter-varietal improvement for grain yield, early maturity, and disease resistance (Harinarayana 1986). For example, using pure-line selection from the germplasm accession, finger-millet culture WWN-25 has been released as a high-yielding variety, GNN-7, for cultivation in Gujarat state of India (Patil et al. 2016). This is a promising development as this variety contains higher Ca (468.0 mg/100 g) than the national check variety VR-708 (398.0 mg/100 g) without compromising on the yield. However, optimum deployment of other breeding methods, such as recombination breeding, for generating stable hybrids, breeding progeny and inbred lines has been delayed due to challenging biparental cross, difficult emasculation and artificial hybridization in finger millet. To overcome these challenges, induced mutations, such as genetic male-sterile systems (viz., INFM 95001 reported by ICRISAT; http://oar.icrisat.org/618/1/PMD_71.pdf) have proved to be another efficient breeding tool for yield and disease resistance in finger millet. These systems and their subsequent breeding can be used effectively to increase the genetic variance by creating new recombinants and segregating populations by exploiting the genetic background. Therefore, developing genetic resources for finger millet, such as mapping populations, breeding lines, and male-sterile mutant lines (Gupta et al. 1997; Krishnappa et al. 2009; Parashuram et al. 2011), deserves attention. Such material will be immensely valuable for tagging nutritional quality traits, especially grain Ca content, and thus facilitate genetic biofortification of finger millet (Puranik et al. 2017).

3.5 Marker-Assisted Breeding

The use of molecular markers in conventional breeding techniques has improved the accuracy of crosses and allowed breeders to produce genotypes with combined traits that were very difficult before the advent of DNA technology. DNA markers can be generated in large numbers and can prove to be very useful for a variety of purposes relevant to crop improvement. RAPD markers were initially the choice by default to characterize finger-millet germplasm. Many efforts made earlier to elucidate the species relationship clearly showed the allotetraploid origin of cultivated species

E. coracana subsp. *coracana*. Direct origin from *E. coracana* subsp. *africana* along with *E. indica* as one of the genome donors was suggested. Low level of polymorphism has been reported in most of the studies on diversity analysis using molecular markers in cultivated finger millet (Muza et al. 1995). For the first time, Dida et al. (2007) developed genomic SSRs by isolating di- and trinucleotide SSRs from random genomic *Hin*dIII, *Pst*I, and *Sal*I libraries of finger millet. They developed first genetic map of finger millet with 31 genomic SSRs as well as RFLP, AFLP, and EST markers. The sudden increase in the volume of sequence data generated from EST projects in several plant species facilitated the identification of genic SSRs in large numbers. Perhaps the most important feature of the genic SSR markers is that these markers are transferable among distantly related species, whereas the genomic SSRs are not suitable for this purpose. Since the genomes of minor grasses like finger millet are yet to be sequenced, for developing markers in minor grasses information can be fetched from the major cereals. Comparative genetic mapping of cereal crops has shown that both gene contents and/or gene orders are largely conserved over the evolutionary history of the grasses (Moore et al. 1995) to the extent that grass genomes represent a 'single genetic system' (Bennetzen and Freeling 1997). Assessment of genetic diversity in finger millet revealed important information that South Indian and the African genotypes are close together and genetically distinct from North Indian genotypes including Uttarakhand (Panwar et al. 2010). Some of these genotypes of this crop possess very high grain Ca^{2+} content (450 mg %) which is 10–30 times higher than wheat and rice (Panwar et al. 2010; Kumar et al. 2012). In a study on Uttarakhand genotypes, molecular marker analysis differentiated the genotypes into three distinct clusters according to Ca^{2+} content indicating that variation in calcium content is also genetically controlled (Panwar et al. 2010). Markers have been utilized extensively for marker-assisted selection, based on their association with genes/QTLs controlling grain Ca^{2+} trait. In order to identify the markers associated with high grain Ca^{2+} trait, 146 genic SSR markers were assessed for cross species transferability across a diverse panel of grass species. The average transferability of genic SSR markers from sorghum to other grasses was highest (73.2%) followed by rice (63.4%) with an overall average of 68.3% which establishes the importance of these major crops as a useful resource of genomic information for minor crops. The genic SSR primers (69.7%) failed to detect variations across the finger millet germplasm, indicating that the mineral transport and storage machinery remain conserved in plants and even SSR variations in them remain suppressed during the course of evolution (Yadav et al. 2014). Development and molecular characterization of genic molecular markers for grain protein and calcium (Ca^{2+}) content have also been done (Nirgude et al. 2014). Of the 86 SSRs used in linkage and association mapping study, only six primers were polymorphic among the two parents PRM 801 and GE 86. Further, 20 polymorphic primers used across the association mapping panel of 238 genotypes led to the identification of five SSR markers, viz., ugep67, ugep24, ugep77, ugep12, and ugep10, which were significantly associated with Ca^{2+} trait. For identifying QTLs for Ca^{2+} content, marker-trait association has been explored through association mapping studies and two minor QTLs associated with grain Ca^{2+} content on linkage group 3 and 8, respectively, have been identified

(Yadav et al. 2014). Linkage group 8 has been found to harbor a minor QTL for the trait and since it has been shown in earlier reports that rice and finger millet share high levels of conserved co-linearity (Srinivasachary et al. 2007) between their genomes it can be speculated that finger millet chromosome 8 might also contain genes/regions responsible for effective mineral accumulation. Similarly, finger-millet LG 3 shares co-linearity with rice chromosome 3 and Ca^{2+} QTLs have also been mapped on chromosome 3 of rice. Furthermore, these results indicate that broad genome-wide search will be required to identify all the genes that control this complex trait and variation in a population. However, the inbreeding nature, limited recombination rates, and a historical genetic bottle-neck during isolated domestication of this crop significantly impact the extent of available genetic diversity in finger millet. Such loss of genetic diversity is a challenge for geneticists and breeders working with a limited number of finger-millet accessions. Further, until recently, there has been no progress in application of the finger-millet genetic map in trait mapping despite the assembly of the only molecular marker-based linkage map a decade ago (Dida et al. 2007; Srinivasachary et al. 2007). It still remains underutilized for tagging and identification of genes/quantitative trait locus (QTL) governing grain Ca^{2+} content probably due to an insufficient number of informative markers. Insufficient number of markers and lack of genome sequence information in finger millet has resulted in limited breeding efforts for nutritional quality traits improvement. However, advances in large-scale genomics technology have now streamlined production of genome-wide markers, which can be used for large-scale identification of candidate gene loci (Puranik et al. 2017).

This advancement has led to SNP discovery in finger millet. Kumar et al. (2016b) where 23,000 SNPs have been identified through genotyping by sequencing (GBS) of 113 diverse finger-millet genotypes. Similarly, 23,285 SNPs were generated using next-generation sequencing of two cultivated finger millet genotypes, and 92 SNP markers were validated further for genetic diversity in cultivated and wild species of finger millet Out of 92, 80 SNP markers were polymorphic. However, SNP markers also resulted in a low PIC value of 0.29 revealing narrow genetic base of finger millet as reported with SSRs (Gimode et al. 2016). In future, these SNPs could be further analyzed to identify useful marker(s) associated with grain calcium (Table 3.3).

3.6 Genomics-Assisted Breeding for Dissecting Complex Trait of Calcium Nutrition in Finger Millet

Finger millet is bestowed with agriculturally and nutritionally important traits such as its adaptive nature, good ability to grow under organic conditions, and high mineral content (calcium, iron, and zinc) and quality proteins. A revolution in the field of omics science will help in understanding the complexity of these traits. The aim of functional genomics is to discover the function of all genes, typically through high-throughput approaches such as genomics, proteomics, or metabolomics

Table 3.3 Molecular marker studies in finger millet for calcium trait

Source for designing primers	Primers	Polymorphism	References
Calcium (Ca^{2+}) transporters and sensors of rice and sorghum	23 anchored EST SSRs	14 polymorphic markers	Kumar et al. (2015)
Calcium (Ca^{2+}) transporters and sensors of rice and sorghum	146 EST SSRs	No polymorphism	Yadav et al. (2014)
Candidate genes, viz., Calcium (Ca^{2+}) exchangers, channels and ATPases of finger millet, rice, maize, wheat, and barley	20 anchored SSRs	5 polymorphic markers	Nirgude et al. (2014)

combined with bioinformatics tools for data analysis and functional analysis. Discovery of gene functions is an essential task in functional genomics; however, it is not sufficient for crop improvement and probably of little use for enhancing selection for quantitative traits such as crop yield. To decode agronomical traits, functional genomics approaches can be of good use for understanding molecular and genetic processes underlying complex traits. Enormous progress has been made in the genomics technology through application of high-throughput, economical, and quicker next-generation sequencing (NGS) platforms. Extending the benefits of NGS to finger millet, a recent effort of de novo sequencing has allowed whole genome sequence assembly covering approximately 82% of total estimated genome size. Evidence of higher colinearity with foxtail millet and rice as compared to other Poaceae species, and the available genome sequencing information may help allele discovery and candidate gene identification for agronomically important traits (Hittalmani et al. 2017) leading to faster development of improved varieties. In addition, GBS, which is a NGS-platform-based highly multiplexed genotyping system, has also been applied for SNP generation. Thus, now it is feasible to generate a higher density of markers by genotyping core collections of finger millet, thereby increasing the level of genetic diversity explored. This is crucial for a predominantly self-fertilized crop like finger millet because it is expected to have low recombination rates and high linkage disequilibrium (LD) which would otherwise narrow the genetic diversity. Thus, the more genetically diverse populations in finger-millet core collections, together with the huge amount of relevant marker information generated through NGS platforms can directly contribute to improved mapping resolution of traits, such as Ca^{2+} content through genome-wide association studies (GWAS). The GWAS studies can confirm previously identified genes involved in Ca^{2+} homeostasis mechanisms as well as spot putative novel candidates. However, the efficiency of GWAS depends upon accurate

grain Ca^{2+} content phenotyping data over multi-location/multi-year trials (Puranik et al. 2017).

An extension of MAS, genomic selection (GS) is an upcoming methodology in the area of genomics-assisted breeding (Meuwissen et al. 2001). In this approach, genome-wide marker genotype data along with available phenotypic data for a tested (reference/training) population are used to predict the performance of an untested (breeding) population based on genomics-estimated breeding values (GEBV). Thus, instead of identifying few large-effect loci associated with Ca content, the GEBV model can more accurately predict the expected phenotype of a broader breeding population. This significantly reduces the time and costs associated with phenotyping a trait like grain Ca^{2+} content. Finger millet enjoys the availability of germplasm resources, such as the core collections, which can be utilized as test populations to build genomic prediction models (Heffner et al. 2010). However, the applicability of GS in finger millet and selection of superior genotypes are dependent upon precise measurement and heritability of Ca^{2+} content, sufficient marker density, the extent of LD decay, effective design of training population and its genetic relationship with the breeding population (Varshney et al. 2014). However, NGS can help in exploring genetic diversity across germplasm sets in depth, thereby, bringing forward a huge wealth of genetic information. This will eventually lead to a new horizon for finger-millet Ca^{2+} biofortification (Fig. 3.1).

3.6.1 Rapid Breeding Cycles: Increasing the Genetic Gain

Recently, the concept of "Speed breeding" has been brought to spotlight which enables scientists to exploit gene bank accessions and mutant collections for rapid gene discovery and gene use. Combining speed breeding and other advanced plant-breeding technologies might help in achieving the genetic gain targets required to deliver our future crops to meet the demands of the global population (Li et al. 2018). However, the recent development of rapid breeding cycles uses extended photoperiods and controlled temperature regimes to achieve rapid generation cycling in fully enclosed growth chambers or glasshouses for large-scale application in crop-breeding programs. This provides a highly flexible platform to achieve rapid generation advancement, irrespective of genetic background, where up to four-to-seven generations per year can be achieved in six crop species including wheat, durum wheat (*Triticum turgidum*), barley (*Hordeum vulgare*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and canola (*Brassica napus*) (Watson et al. 2018). Similar approach could be exploited in finger millet where not only populations could be developed rapidly for genetic studies but also introgression of favorable alleles into elite germplasms could be done for crop improvement by reducing the generation time.

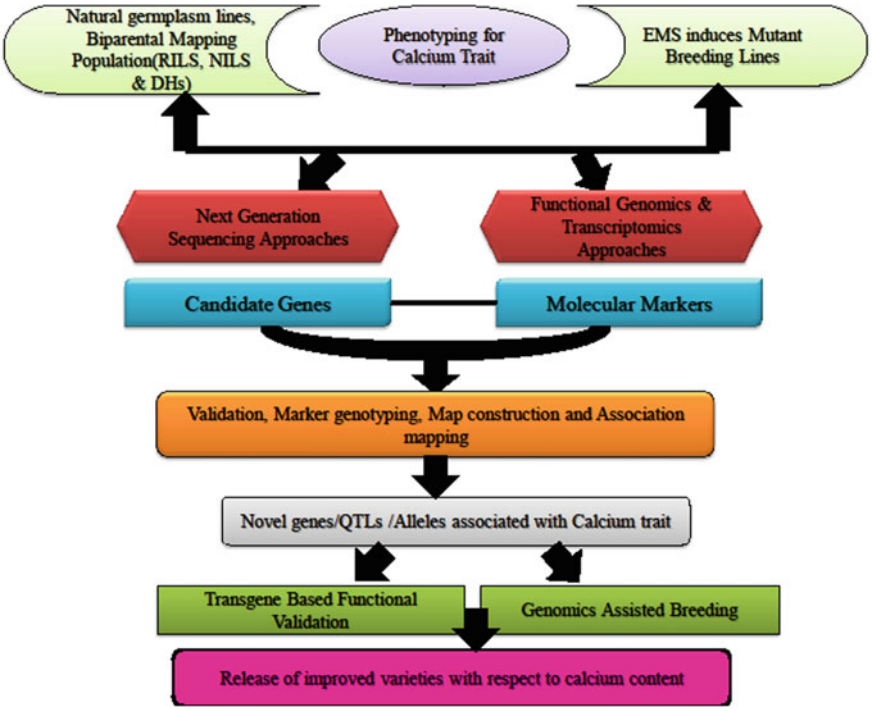

Fig. 3.1 Steps involved in generating calcium biofortified crops using genomics-assisted breeding approaches

3.7 Future Prospects and Conclusion

By virtue of its health-benefitting properties and environmental sustainability, a traditional but minor crop like finger millet offers excellent opportunities for biofortification breeding. Genomic information has not only helped in the understanding of structural and functional aspects of many plant genomes but also provided a feasible platform for the manipulation of genomes for crop improvement. In recent years, sequence information has become readily available for a variety of crop species, but minor crops such as finger millet still lag behind. A foremost priority from geneticists and breeders viewpoint is capturing and utilizing genetic diversity for Ca^{2+} content in the elite finger millet gene pools (for example, by bringing new sources of variation through rare and unique alleles). For trapping such useful variations, advances in the next-generation sequencing technology must be utilized in generating sufficient number of markers for characterizing marker-trait associations and genomics-assisted breeding. With the advent of such high-throughput approaches, it will be much easier to investigate the genetic architecture of this trait through comparative genomics in other millets and non-millet species. Discovery of markers tightly linked to other traits governing grain Ca^{2+} content and identification of underlying genes

could be another strategy to develop Ca^{2+} biofortified finger millet varieties through traditional or modern breeding approaches and transformation-based technology.

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Chapter 4
Iodine Biofortification of Crops

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Abstract The biofortification of crops with iodine consists of a set of techniques that allow obtaining plant foods with concentrations of iodine that partially or entirely provide the daily intake requirements for humans. Iodine is transferred from plants to humans through the trophic chain, which is why we seek to biofortify the crops as part of the strategy to ensure adequate consumption of this element. The concentration of iodine in food depends primarily on the ability of plants to absorb and accumulate it, as well as the capacity of soil and water to provide it in bioavailable forms for plants. In many soils, the low concentration of bioavailable iodine is the result of intrinsic geological factors, although in other cases it results from edaphic fixation. These edaphic factors that modify the bioavailability of iodine are well known, pH and ORP, organic matter, minerals of colloids, and microbial activity. Although almost all of these factors are or can be part of routine agronomic management, little is known about their proper combinations to increase the bioavailability of iodine that is fixed in agricultural soils. All of the experimental reports on biofortification refer to the contribution of exogenous iodine in mainly inorganic (KI and KIO_3) and some organic (kelp and iodinated organic acids) forms both in soil crops and in soilless production systems. The ideal situation would be for exogenous applications to be used mostly for crops in soilless systems, whereas for the crops in soil, the exogenous applications were a complement to agronomic management that promoted the bioavailability of iodine in the soil solution. As iodine is not an element qualified as essential for plants, the extension of its use among agricultural producers will

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not be simple, unless it ensures some utilitarian facet of iodine, which could be its antioxidant capacity and potential inducer of tolerance to stress.

Keywords Nutritional quality · Nutraceutics · Functional foods · Iodine deficiency disorders · Trace elements in plants · Organic iodinated compounds · Beneficial elements in plants

4.1 Introduction

Iodine is considered one of the first antioxidants used by living organisms. In the marine environment, iodine is available in dissolved forms and is absorbed and concentrated by photosynthetic organisms that in turn transfer it into the trophic chain. It is believed that when different organisms evolved to live in terrestrial areas (where the availability of iodine is much lower than in the sea), dependence on iodine changed. In terrestrial plants, a whole new set of antioxidants (such as ascorbic acid, polyphenols, and carotenoids) emerged to replace iodine (Venturi 2011). It is possible that this fact partially explains the low concentration of iodine in the structures of terrestrial plants compared to marine photosynthetic organisms. Although it has not been demonstrated that iodine is an essential element for terrestrial plants, it is known that plants absorb it by roots and leaves and dissipate it into the atmosphere in gaseous form (Barry and Chamberlain 1963; Whitehead 1979; Amiro and Johnston 1989) using non-vanadium-dependent halogen methyltransferases (Landini et al. 2012), so it is possible that iodine performs metabolic functions not yet well understood in terrestrial plants (Gonzali et al. 2017).

In terrestrial animals, iodine is an essential element, therefore in response to the reduced availability of iodine out of the sea during the evolutionary process proteins or tissues, such as the thyroid follicles, specialized for the storage of the element, were developed (Venturi 2011). Iodine, stored in proteins or specialized structures of animals is obtained mostly from food intake and, to a lesser extent, the absorption of iodine from drinking water and air that reaches the lungs (Vought et al. 1970; Whitehead 1984; Fuge and Johnson 2015).

Iodine is one of the most studied elements due to its metabolic importance in humans and the complexity associated with the factors that induce its deficiency. According to the World Health Organization (WHO), the most common nutrient deficiencies are iodine (Fig. 4.1), along with those of iron (Fe), zinc (Zn), and vitamin A (Burlingame 2013; Prasad 2013).

The irregular distribution of iodine in the Earth' crust correlates with its different regional availability in soil and water and results in the deficiency of this element in various regions of the planet (FAO 2009). It is estimated that around 2 billion people live with an insufficient iodine intake (Mottiar 2013), causing what is known as iodine deficiency disorders (IDD). The IDD is the metabolic and developmental abnormalities associated with low iodine intake and can be prevented by ensuring adequate intake of the element (Zimmermann et al. 2008).

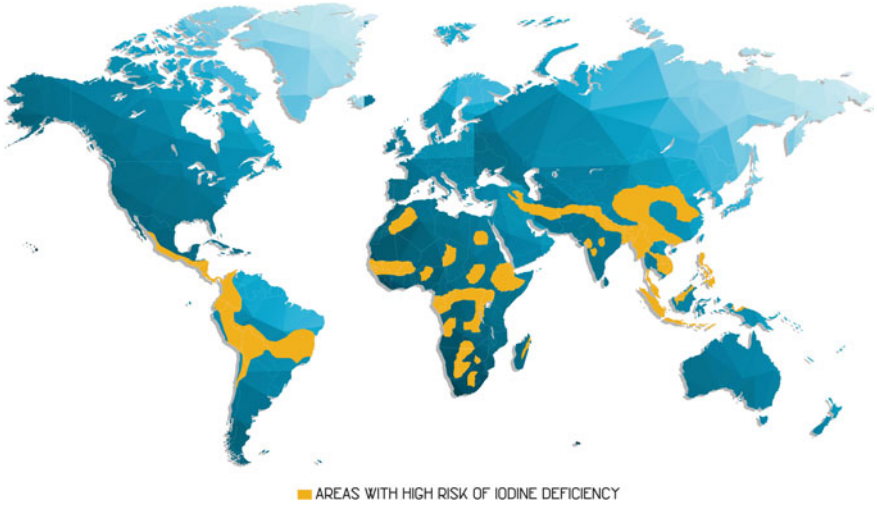

Fig. 4.1 On the map, the regions where the human population presents a higher risk of iodine deficiency are indicated in orange (Medrano-Macías et al. 2016a)

The most recognizable IDD is goiter. However, in the last decades, the presence of less tangible IDD has been evidenced. Examples are the higher incidence in fetal death, miscarriages, and congenital anomalies (Zimmermann 2009), as well as the adverse impact on mental and physical development during childhood (Manousou et al. 2018), and in the labor productivity of adulthood (Lazarus et al. 2012). Similarly, the action of iodine as an antioxidant and as antiproliferative of malignant cells was recently demonstrated (Funahashi et al. 2001; Aranda et al. 2013; Anguiano and Aceves 2016). The daily iodine requirements at different stages of human development were described by (World Health Organization 2007; Andersson et al. 2012) and are presented in Table 4.1.

Depending on the source, conservation, and preparation, foods from animal and vegetable sources contain different amounts of iodine. In an extensive study of the literature, (Fordyce 2003) indicated that the geometric mean of the concentration of iodine in various types of food is 87 μg (kg food)$^{-1}$, a low value when considering the data in Table 4.1. The breakdown of the iodine content by type of food is indicated in Table 4.2.

Table 4.1 Recommended daily iodine intake (Medrano-Macías et al. 2016a)

Daily intake (μg)	Age
90	Infants (0–59 months)
120	Children (6–12 years)
150	Adults (older than 12 years)
200	Pregnancy and lactation

Table 4.2 Iodine content in different foods

Food	Concentration μg I (kg food)$^{-1}$
Saltwater fish	1455.9
Freshwater fish	102.8
Leafy vegetables	88.8
Dairy	83.9
Other vegetables	80.1
Meats	68.4
Cereals	56.0
Fresh fruits	30.6
Drinking water	6.4

Modified from Medrano-Macías et al. (2016a)

Except for the sea fish, the rest of the foods showed low average values of iodine. It is expected that the data displayed in Table 4.2 show variation between sites and seasons of harvest or production. However, the need to increase the amount of iodine in food is pointed out. For example, in lettuce, it has been suggested to raise the concentration to 50–100 μg of iodine per 100 g of fresh weight (Lawson et al. 2015). In contrast, in a study carried out on cereals from continental agricultural areas in Europe, the iodine concentration was particularly low, with values based on the dry weight from 2 to 30 μg kg^{-1} (Shinonaga et al. 2001). Among the alternative food sources of iodine should be highlighted marine algae, such as kelp (of the genus *Laminaria*). Kelp accumulates iodine with values of 0.25–1.20% of its dry weight (Gall et al. 2004). Another alternative is some freshwater microalgae that reach iodine up to 0.04% of its dry weight (Han et al. 2016). Numerous attempts have been made to mitigate the deficit in iodine consumption among the human population. One technique that has proved effective is salt fortification (iodization) that began in the 1920s (de Caffarelli 1997; Zimmermann 2009; Charlton et al. 2013). However, it has been found that the fortification of table salt alone is insufficient to ensure the iodine requirement (de Benoist et al. 2008) because inorganic iodine added to the salt is lost by sublimation, especially in warm places (Mottiar and Altosaar 2011). On the other hand, the consumption of iodine in organic forms such as those found in algae, fungi, and biofortified terrestrial plants is more bioavailable (Rakoczy et al. 2016) and is considered more effective in preventing IDD (Funahashi et al. 2001; Weng et al. 2008a; Li et al. 2018). Part of the explanation of the higher dietary effectiveness of organic sources of iodine is that the rate of loss from volatilization is very low, since iodine has a high capacity to form complexes with polymeric materials (Moulay 2013; Limchoowong et al. 2016) as in vegetable starches (Mottiar and Altosaar 2011) and waxy cuticle materials (Tschiersch et al. 2009). Due to the above, it is necessary to promote the use of biofortification of crops to achieve an adequate consumption of iodine, either as a supplement or as an alternative to the iodization of table salt. The objective of this chapter is to show the progress made in obtaining

crop plants biofortified with iodine, showing the different techniques used and the results obtained.

4.2 The Presence of Iodine in Plant Foods, Soil, and Water

The largest reservoir of iodine on the planet is the ocean, with a concentration of 50–84 $\mu g\ L^{-1}$ (Schnepfe 1972), with large variations in the vertical profile and between different latitudes. From marine waters, iodine is volatilized into the atmosphere in the form of organo-iodinated compounds (CH_3I and CH_2I_2) and molecular iodine (I_2) (Carpenter et al. 2000). The volatilization is carried out by infinity of marine organisms: bacteria, microalgae, and macroalgae such as those of the genus *Laminaria* (Leblanc et al. 2006). In *Laminaria*, this process is carried out through a mechanism related to oxidative stress and H_2O_2 production (Küpper et al. 2008), linked to the function of the vanadium-dependent iodoperoxidase enzyme (V-IPO).

In the atmosphere, iodinated compounds are photolyzed and react with ozone molecules to form new chemical species, such as iodine monoxide, dioxide, trioxide, tetroxide, and pentoxide (IO, I_2O_2, I_2O_3, I_2O_4, and I_2O_5). The result is the formation of condensation nuclei for cloud formation (Saunders and Plane 2005). Subsequently, the incorporation of the element will occur on the soil by means of rain and snow, with an iodine concentration of 0.5–2.5 $\mu g\ L^{-1}$, mainly in the form of iodide (I^-) and iodate (IO_3^-) (Fuge and Johnson 1986). The transfer of atmospheric iodine to the earth surface also occurs by dry deposition (Baker et al. 2001), but incorporation associated with precipitation is considered the most crucial type of transfer (Whitehead 1984). Gaseous iodine in its elemental state (I_2) is most susceptible to deposition by rain or snow, whereas methyl iodide (CH_3I) is less susceptible (Slinn 1978). Dry deposition occurs by gravity sedimentation and is related to wind and turbulence (Whitehead 1984).

As a result of the transfer of the sea to the continental zones, it has been observed that the soils that are located at a distance of between 50 and 80 km from the ocean have an iodine content higher than those that are furthest away (Fuge and Johnson 2015).

The global cycle of iodine (Fig. 4.2) is completed by returning from the soil to the atmosphere, through biotic or abiotic processes, or dragged to the ocean through groundwater (Moreda-Piñeiro et al. 2011).

Iodine is subject to a complex dynamic that determines its availability. There is a balance between fixation and volatilization depending on the physicochemical characteristics of the soil, such as the content of organic matter, interaction with other minerals, the action of the soil microbiome, pH, and oxidation-reduction potential. Consequently, the concentration of iodine in the soil is very variable, reporting a range of <0.1–150 $mg\ kg^{-1}$, with the global average of 5.1 $mg\ kg^{-1}$ and a geometric mean of 3.1 (Johnson 2003).

The iodine that remains in the soil can be found in bioavailable form in the soil solution as IO_3^- or I^- or it can be fixed in non-bioavailable complexes with organic

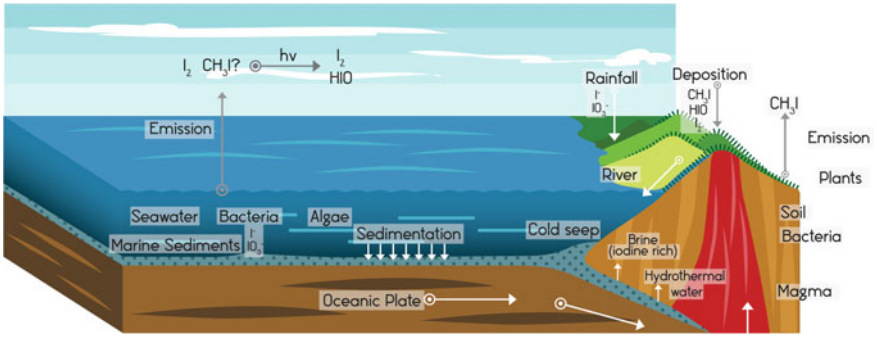

Fig. 4.2 Global iodine cycle. Figure of (Medrano-Macías et al. 2016a)

matter, clays, and the oxides of Fe and Al (Fuge 2013), with the latter particularly in soils with acid reaction (Whitehead 1974; Heumann et al. 1990). The adsorption of IO_3^- by the soil components is 2.6 times higher than that of I^- (Kodama et al. 2006; Lawson et al. 2015), but the relative amount of IO_3^-/I^- depends on the redox and pH conditions. Most of the non-bioavailable iodine is retained in the first 10 cm of the edaphic profile (Weng et al. 2009; Yoshida et al. 2007). The bioavailable forms IO_3^- and I^- can be leached or volatilized as hydrogen iodide (HI) or iodic acid and periodic acid (HIO_3, HIO_4) (Sheppard et al. 1994).

Organic matter plays a central role in the retention of iodine in the soil (Whitehead 1973; Dai et al. 2006). It has been established that with a high content of organic matter, low iodine volatility occurs, presumably due to the formation of covalent bonds with carbon atoms (Moulin et al. 2001; Schlegel et al. 2006; Stavber et al. 2008). Aerobic conditions increase the iodination of soil organic matter, a process that is enhanced by microbial activity (Yeager et al. 2017). It has been shown that the I^- form is more susceptible to reacting with organic matter, through a mechanism of electrophilic substitution of a proton by an iodine atom in the aromatic rings in humic and fulvic substances (Muramatsu et al. 2002). Almost all the organic iodine is associated with humic acids (63%) and in lesser quantity with fulvic acids (20%) (Yamada et al. 1999).

The oxidation of I^- favors the fixation in organic compounds. In this context, it is known that H_2O_2 can increase the rate of formation of organo-oxidized compounds in soil, substrates, and sediments. H_2O_2 is produced abiotically by the organic matter subjected to UV radiation and soil microorganisms. H_2O_2 converts I^- to HOI and I_2, which react with fulvic acids. The IO_3^- reacts with H_2O_2 forming reactive iodine species that are associated with lignins, tannins, and carboxylic compounds of soil organic matter. It has been found that organo-iodinated molecules of the soil can be proteinaceous, unsaturated hydrocarbons, and lignin (Xu et al. 2013). It has also been reported that the I^- is fixed in organo-iodinated compounds by microbial oxidases (Seki et al. 2013) and by the action of organic acids secreted by bacteria (Li et al. 2012).

Oxidation of organic matter releases the iodine from the organo-iodinated molecules, exposing iodine to leaching and volatilization. Under aerobic conditions, the presence of Fe_3^+ induces the formation of volatile alkyl halides such as iodomethane, iodoethane, 1-iodopropane, and 1-iodobutane (Keppler et al. 2000). The processes described are modified by the action of the soil microbiome, which can increase the rate of iodine volatilization by oxidation of I^- to CH_3I, HIO, and I_2 (Amachi et al. 2003; Seki et al. 2013).

Soil minerals can be present as free ions, ions adsorbed on organic surfaces, or in mineral colloids, as dissolved or precipitated compounds or incorporated into the microbial biomass (White and Broadley 2009). The oxides and hydroxides of Al, Fe, and Mn are linked by weak electrostatic attractions to the iodate (IO_3) decreasing their bioavailability but also avoiding leaching and volatilization (Shetaya et al. 2012; Tolu et al. 2014). In contrast, I- shows less affinity for soil minerals, which facilitates their mobility and bioavailability, but also their volatilization (Kaplan et al. 2000). In general, the higher active surface of the soil means more capacity to fix the iodine (Faridullah et al. 2017). Therefore, the type of soil influences the mobilization and volatilization of iodine (Weng et al. 2008c).

The associations of iodine with organic matter and soil minerals are not permanent, as they are susceptible to the oxidative changes that the soil undergoes (Yeager et al. 2017). An example of this is what is observed in flooded soils, where desorption occurs and then volatilization occurs in the form of organo-iodinated compounds such as CH_3I (Kodama et al. 2006), contrary to what is found in soils with oxidizing properties and high pH.

Finally, it is known that the chemical species mostly found in reducing conditions (soils with large amounts of organic matter or flooded) and pH < 7 will be iodide (I-) and oppositely with pH > 7 and oxidant condition (soils with low organic matter) or low humidity), it will be the IO_3^- (Dai et al. 2009; Nakamaru and Altansuvd 2014). When comparing the amount of bioavailable iodine under one condition or another, it was found that the concentration of I- was slightly higher under reducing conditions than that of IO_3^- under oxidizing conditions (Yuita 1992).

Regarding the surface water, the concentration of iodine commonly ranges from 0.5 to 5 $\mu g\ L^{-1}$, however, in some cases it can reach about 20 $\mu g\ L^{-1}$ (Watts et al. 2010). These variations depend mainly, like the concentration of iodine in the soil, on the proximity to the ocean. However, other factors have shown a strong correlation such as the geology of the soil through which rivers flow. For example, a higher concentration was found in sedimentary rock than in igneous rock (Fuge and Johnson 1986) as well as in carbonate rocks compared to non-carbonated rocks (Korobova 2010). On the other hand, in the underground aquifers, the reported concentrations are generally higher. In some places, values as high as 430–14,500 $\mu g\ L^{-1}$ are found, probably due to the desorption from organic matter rich in iodine, sediment intrusion, marine or residual saline water in the aquifer or intense evapo-concentration in arid zones (Fuge and Johnson 2015; Voutchkova et al. 2017). Thus, due to the uneven distribution in soils and water, as well as the interactions of iodine with soil components, the bioavailability of iodine to be absorbed and metabolized by plants is conditioned to the area where the vegetation grows. A compilation of the content

Table 4.3 Natural concentration of iodine in water, soil, and plants for human consumption

Concentration in soil (mg kg^{-1})	Concentration in plants (μg kg^{-1})	References
Concentration of Iodine in soils and agricultural plants		
3.35 forest zone in Russian plane	Gramineae 128, *Leguminosae* 121	Korobova (2010)
11.8 andosol upland in Japan	Wheat and Barley 10–100	Uchida and Tagami (2011)
0.66 agricultural soils in Pakistan, but only 2.4% of that iodine was bioavailable	10 in wheat grain, but available iodine was below the detection limit	Watts et al. (2015)
1.6 in archived samples of the last 105 years of an experimental field in Rothamsted, UK	112–285 in herbage samples	Bowley et al. (2017)
0.13–10 in agricultural soils with high clay content	Rice 58 in Sri Lanka	Fordyce et al. (2000)
54 in agricultural soil from the coastal region in Malawi	Staple crops 1, tubers and roots 8, leafy vegetables 155	Watts et al. (2015)
Concentration in water (μg L^{-1})	Concentration in plants (μg kg^{-1})	References
Concentration of iodine in water for agricultural use and plants		
7.3 in agricultural waters in Pakistan	Wheat grain 10, but available iodine was below the detection limit	Zia et al. (2015)
84 drinking water in Wet Zone of southwest Sri Lanka	Rice 58 in Sri Lanka	Fordyce et al. (2000)
52 in drinking water in the coastal region in Malawi	Staple crops 1, tubers and roots 8, leafy vegetables 155	Watts et al. (2015)

of iodine in soil and water, as well as its presence in plant species, is presented in Table 4.3. The concentration in cereals goes from 1 to 128 μg of I per kilogram of grains, whereas the recommendation of daily consumption of this element for an adult is 150 μg and the intake of these grains is approximately 50 g day^{-1} (WHO 2009). However, it has also been reported that in places close to the coasts, andosol-type soils can be rich in iodine and produce grain crops with an adequate iodine level (Uchida and Tagami 2011).

4.3 Iodine Uptake by Plants

Iodine is an element that can be absorbed through the cells of the epidermis of the root, as well as through the stomatal pores and the cuticle of the epidermal cells of the leaves. Of the total iodine absorbed by the plants, it is not known how much on average it comes from the soil and how much of the atmosphere. It is expected that

the atmospheric contribution will be more significant in the regions near the coast and lower in the continental areas, far from the sea and with soils with a high level of iodine fixation (Whitehead 1984; Fuge and Johnson 2015).

The absorption of iodine by the roots of plants depends on the amount of bioavailable iodine (I^- and IO_3^-) present in the soil solution. Both chemical species can be absorbed by the roots, but there seems to be a specific preference for I^- (Blasco et al. 2008; Mackowiak et al. 2005; White and Broadley 2009). The suggested explanation is that the root cells can absorb both forms of iodine, but that part of the IO_3^- of the soil solution or nutrient solution is reduced to I^- through of an iodate reductase that responds to the availability of iodine in the substrate. The iodate reductase activity is not detected in the absence of iodine, shows high activity in the presence of IO_3^-, but decreases with the excess of iodine (Kato et al. 2013). This reductase activity also occurs in microorganisms (Amachi 2008), but the magnitude of the microbial contribution to this process is unknown. On the other hand, a substantial difference occurs in the mobilization of iodine according to whether it is given to the roots in inorganic (KI) or organic form (5-iodosalicylic acid, 5-ISA; 3,5-diiodosalicylic acid, 3,5-di-ISA; 2-iodobenzoic acid, 2-IBeA; 4-iodobenzoic acid, 4-IbeA), finding that the KI is quickly mobilized to the aboveground parts of the plant, while the I in organo-iodinated substances accumulated mainly in the roots (Halka et al. 2018).

It has been proposed that iodine transport occurs through chloride channels (Mottiar and Altosaar 2011). Mainly for the I^-, the plants seem to absorb it and mobilize it through the organs of the plant using anionic channels and chloride transporters energized by proton pumps (White and Broadley 2009). The identity of I^- transporters is not firmly established, but it is assumed that the activity can be shared by several families of transporters and anion channels, including organic acid transporters (White and Broadley 2009, 2001; Landini et al. 2012). As a consequence, the presence of anions such as nitrate, thiocyanate, and perchlorate can interfere with the absorption of iodine (Voogt and Jackson 2010). The transport of iodine to fruit and seeds through the phloem does not occur as efficiently as in the xylem. But despite this, its presence is reported in different organs of the plants that receive a significant flow of the phloem, including fruits and seeds (Mottiar and Altosaar 2011; Kiferle et al. 2013; Kopeć et al. 2015; Smoleń et al. 2016a; Cakmak et al. 2017).

4.4 The Flux of Iodine in the Plant System

Once the root cells absorb I^-, the iodine is believed to be mobilized in the plant using H^+/anion symporters and anionic channels that load it in the xylem (White and Broadley 2009). From the root to the stems and leaves, the flow by xylem seems to be the predominant form of iodine transport (Dai et al. 2004; Zhu et al. 2003), with the lowest redistribution by the phloem (Herrett et al. 1962). That is why iodine accumulates in greater quantity in the leaves compared to fruits and seeds (Gonzali et al. 2017). For example, in the study carried out by (Haldimann et al. 2005), it was found that iodine from food samples had values (in $\mu g\ kg^{-1}$ of dry weight) of 35 in

wheat, 333 in rice, 16 in potatoes, 18 in fresh fruit, 47 in fresh vegetables, and 236 in a salad with leafy vegetables.

In studies where the flow of iodine is followed through the different structures of the plant, isotopes of iodine are used, such as the case of ^{125}I, which has been found in higher proportion in root > stem > petiole > leaf (Weng et al. 2009). Similarly, in an analysis of I^- and IO_3^- made in strawberry plants, a higher concentration of iodine was found in roots > leaves > stems > fruits, with the higher concentration of iodide compared to iodate (Li et al. 2017b).

To quantitatively describe the ability to mobilize iodine from the soil, substrate or nutrient solution to the organs that are harvested, the iodine transfer factor (ITF) is used, which is defined as the quotient between the concentration of iodine in plant tissues and the concentration in the substrate. The ITF is then higher in species that are grown to produce leaves, such as spinach (ITF $\geq$ 2.0), than in tomatoes, wheat, and other species where fruits and seeds are harvested (ITF between 0.0005 and 0.02) (Lawson 2014). ITF does not constitute constant values; it changes in response to environmental stimuli and its value increases when plants have bioavailable iodine in the soil solution, nutrient solution, or by foliar spray (Dai et al. 2004; Smoleń et al. 2017; Cakmak et al. 2017).

Once the iodine is transported and is present in different organs of the plants, it is unstable and can be volatilized by enzymes called halide ion methyltransferases (HMT) and halide thiol methyltransferase (HTMT) dependent on S-adenosylmethionine (Itoh et al. 2009; Medrano-Macías et al. 2016a). Volatilization can occur with iodine in the soil by roots and microorganisms, but in the case of rice, the highest volume of volatilization happened in the stems and leaves of plants in the form of CH_3I (Muramatsu and Yoshida 1995). It was shown that volatilization incessantly decreases the store of iodine present in plants (Landini et al. 2012; Gonzali et al. 2017). The importance of volatilization activity was demonstrated by obtaining arabidopsis plants with increased accumulation of iodine by knocking down the HOL-1 gene which encodes a halide methyltransferase (Landini et al. 2012). The metabolic activity of iodine volatilization by plants contributes to the general activity of volatilization that occurs in soils and inland waters and is part of the global flow of iodine. There is little information about the difference between plant species and environmental factors that affect the activity of enzymes that dissipate iodine. However, it is known that in aqueous solution H_2O_2 is a compound that increases the speed of iodine volatilization reactions. This fact can be a partial explanation of the observations that indicate an increase in the volatilization of iodine in bacteria under stress conditions (Medrano-Macías et al. 2016a). Figure 4.3 presents a resume of the flux of iodine in plants.

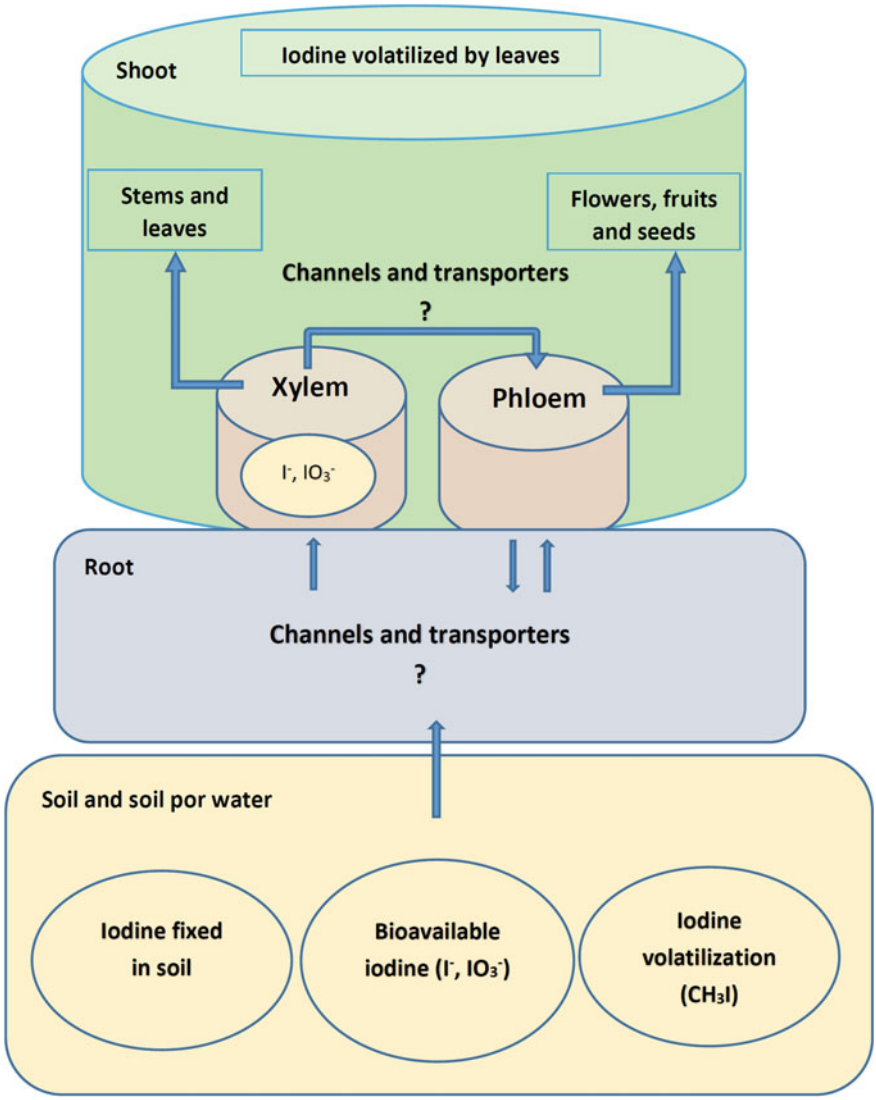

Fig. 4.3 Flux of iodine from soil to plant and atmosphere

4.5 Agronomical Practices to Improve Iodine Absorption and Assimilation in Plants

In the previous sections, information was presented to point out the impact of the various factors of soil, water, and atmosphere on the bioavailability of iodine for plants. This set of factors (soil organic matter and soil microbiome, moisture level, salinity, concentration of elements added with fertilizers, among others) is subject to direct or indirect manipulation in agricultural production systems, thus modifying the availability of iodine in the dissolved forms I^- and IO_3^- found in the soil solution, from where they are absorbed by the roots of the plants. Similarly, agricultural practices such as tillage and the application of vegetable or animal waste, composts, and humic substances modify the oxidation-reduction status of the soil, possibly inducing changes in the relative amounts of iodine that is fixed versus the volatilized. These changes probably alter the amount of iodine in the form of I_2 and CH_3I, which inevitably has an impact on the fixation of gaseous iodine in the aboveground vegetal structures by stomata and the waxy cuticles of leaves, stems, and fruits.

The points described in the two previous paragraphs have been little studied, but it has been suggested that the iodine fixed in the soil can be mobilized applying good agronomic practices, thus raising the concentration of iodine in the crops (Stewart et al. 2003). The alternatives or complementary approaches that have been proposed are the obtentions of biofortified cultures using traditional breeding techniques, the creation of transgenics, and possibly genetic editing (Mottiar 2013). In most published studies, the approach given to the biofortification of crops is based on the exogenous application of iodine in different chemical, organic or inorganic forms, either to the soil, irrigation water, nutrient solution, or by spraying plants.

The most studied method for crop biofortification is the application of inorganic iodine salts. The plants absorb the iodate (IO_3^-) and iodide (I^-) ions dissolved in the soil solution or the nutrient solution using transporter proteins mentioned in Sect. 4.3 of this chapter. When applied to the soil, IO_3^- is a more effective source of iodine compared to I^- (Lawson et al. 2015). On the other hand, I^- can be very useful for biofortification when applied in the right quantities (10–100 μM KI applied biweekly to the substrate or leaf spray), besides that the I^- increases the accumulation of antioxidants in plants (Cortés-Flores et al. 2016; Medrano-Macías et al. 2016b). The disadvantage of I^- is that it generally induces greater toxicity in plants compared to IO_3^-, especially in soilless crops (Borst Pauwels 1962; Umaly and Poel 1971; Muramatsu et al. 1983; Zhu et al. 2003), although (Kiferle et al. 2013) in tomato with four applications of iodine, once a week, observed greater toxicity when applying KIO_3 in the nutrient solution compared to the KI. The concentration recommended by the authors to obtain biofortified tomatoes without damage to the leaves was 2 mM (332 mg L^{-1} KI, 428 mg L^{-1} KIO_3) and could rise to 5 mM for the KI but obtaining slight leaf damage. The muskmelon crop tolerates up to 1 mM KI (166 mg L^{-1} KI), biweekly by leaf spray or weekly in fertilizer solution applied to the substrate (Gordillo-Melgoza et al. 2016), showing a decrease in biomass whit 2 mM. The I^-

and IO_3^- were equally effective for the biofortification of wheat grains by applying 20 mg I kg $soil^{-1}$ or 0.1–0.25% (w/v) (1000–2500 mg L^{-1} of KI or KIO_3) using only three foliar sprays at heading stage, early milk, and initial dough stages (Cakmak et al. 2017).

The lower toxicity of IO_3- may occur because the iodate functions as an alternate substrate for other important enzymes such as nitrate reductase (Barber and Notton 1990) or because the activation of iodate reductase by IO_3^- induces some other responses associated with the metabolism of iodine in plants, in addition to the mere reduction in IO_3^-. The IO_3^- under aerobic conditions is a more stable form than the I^-. Thus, it is likely to be the most abundant bioavailable form in the water of the pores of agricultural soils subject to tillage (Medrano-Macías et al. 2016a).

The localized application of inorganic salts of iodine, using leaf or fruit spray or in the substrate or nutritive solution, in a specific stage of the development of the plants as in seed, seedlings, stems, leaves, or fruits in pre-harvest or post-harvest (García-Osuna et al. 2014; Cortés-Flores et al. 2016; Limchoowong et al. 2016; Jerše et al. 2017; Gonzali et al. 2017) has also been successful. The advantage of the localized applications in a single stage of the development of the plant, or in post-harvest, is that the complexities of applying the iodine continuously are avoided, both in the nutritive solution and to the soil, where some edaphic factors can fix it, limiting the absorption by plants (Smoleń et al. 2016a). Additionally, iodine can be associated with mineral compounds such as hydroxyapatite and diatomite, in biopolymers or complexes of nanomaterials and biopolymers that can be applied to the substrate or soil, decreasing both the fixation by the soil and the losses by leaching or volatilization (Weng et al. 2013; Liu et al. 2014; Weng et al. 2014; Medrano-Macías et al. 2016a). Once the roots of the plants make contact with the materials, the elements contained in the structure of the material can be absorbed by the plants. It should be noted that there is practically no published research on the use of nanomaterials or nanoparticles to improve the availability of iodine for plants.

The issue of volatilization control is considered significant not only from a technical point of view but as part of the promotion of the use of biofortification with iodine, which implies modifying the perception that it is a volatile component that will be lost in if added to fertilizers (Olum et al. 2018). Figure 4.4 shows a summary of different techniques used for biofortification of plants using exogenous iodine application. The authors (Medrano-Macías et al. 2016a) propose to reduce the loss by volatilization of iodine applied using organic iodine sources (such as kelp) to be applied directly to the soil or as a compost, or iodine salts (KI and KIO_3) fixed in biopolymers. In the same way, the use of KI and KIO_3 using foliar spray allows fixing the iodine in the hydrocarbons of the cuticles, decreasing the loss due to volatilization. An additional advantage of foliar spraying is to avoid the complexity of factors that allow or limit the bioavailability of iodine in the soil.

The previously described biofortification techniques using exogenous iodine sources are applicable in virtually all plant species. However, leafy vegetables such as lettuce and spinach are especially useful because of their high ITF (Lawson 2014). Yet, to have a higher reach among the world population, it is necessary to significantly increase the availability of iodine in basic grains such as corn, wheat, and rice

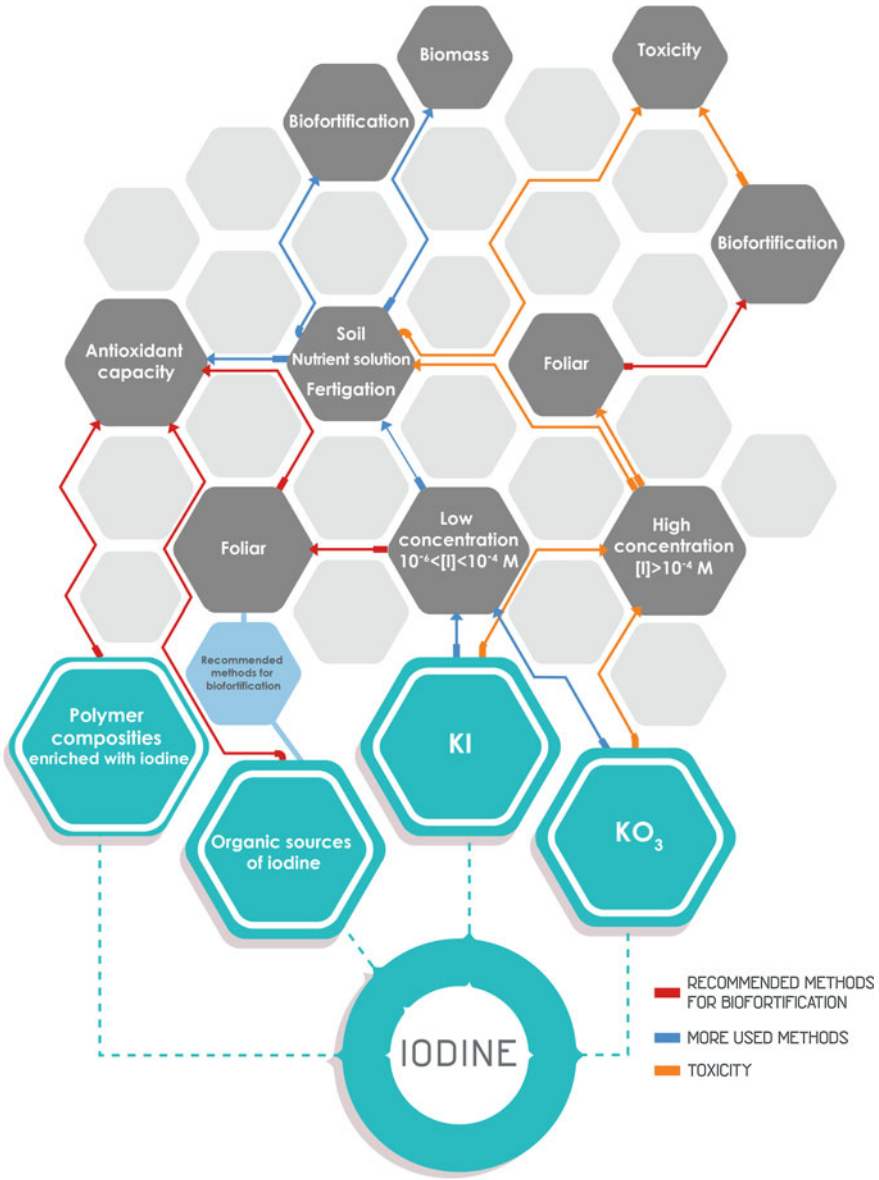

Fig. 4.4 Graphic view of different methods of application of iodine. The left side, with red directional arrows, includes most promissory techniques that use organic sources of iodine applied to the soil, the inorganic iodine for plant spraying, and the use of iodine fixed in polymers for application in soil and substrates. On the right side, the most used methods for the addition of KI and KIO_3 to soil or nutrient solution. The concentration used defines the outcome, the blue arrows indicate a low level of iodine with satisfactory results on biofortification and plant growth. The orange arrows denote a high concentration of iodine that may lead to great biofortification but accompanied by plant toxicity (Medrano-Macías et al. 2016a)

(together, cereals constitute 80% of the vegetable foods produced by the humans and account for 60–75% of calories consumed), for which it is necessary on the one hand to explore the impact of soil management and irrigation practices on the bioavailability of iodine present in the soil, select the best techniques, chemical forms and concentration of iodine for application in plants and, on the other hand, increase the capacity of these species to mobilize iodine to the grains (Cakmak et al. 2017).

It is also advisable to explore much more the possibility of using as options for biofortification other basic crops, such as legumes (Jerše et al. 2018), or crops with a high content of carbohydrates in roots, tubers, or stems such as potatoes and tapioca (*Manihot esculenta*). These last species can be attractive for biofortification since, on the one hand, it is more straightforward to accumulate iodine in roots and stems, and on the other hand, the starch reserves of the plants constitute stable iodine storage (Mottiar and Altosaar 2011; Cerretani et al. 2014).

The impact of iodine on growth and yield is an important component for biofortification. In general, excessive I applications produce a decrease in biomass and yield of fruit or grain; causing, however, through a concentration effect due to the lower biomass obtained, a higher concentration of iodine in the plant parts (Cakmak et al. 2017). In Fig. 4.4, this effect is shown on the right side. Following, Table 4.4 presents some results of studies where iodine was applied to biofortify crops. The included results refer to different crop species where different sources and concentrations of iodine were applied to the soil, substrate, irrigation water, nutrient solution, or by foliar spray. The information is useful as a reference for the design of biofortification strategies in the field or greenhouse.

Table 4.4 shows a great diversity of situations concerning the form of application, concentration, and chemical species used. Each species and probably the cultivars or ecotypes within a species are tolerant to a specific range of concentrations of iodine that allows biofortification and even becomes beneficial, while higher amounts cause toxicity. The threshold between benefit and toxicity will be different in each species, due to the action of intrinsic variables to plants, as well as edaphic, climatic, and biotic interaction factors (Hageman et al. 1942; Mackowiak et al. 2005; Caffagni et al. 2012). Below is a summary of recommendations about the application of iodine in agricultural systems.

Recommendation for the application of iodine. The global average of the concentration of iodine in the soils is 2.6 mg kg soil^{-1} (Watts et al. 2010). The contributions up to 10 mg kg soil^{-1} favor plant growth with a good result in biofortification. The use of more than 50 mg kg soil^{-1} significantly raises the iodine concentration in plants, but in some species, it will produce an adverse effect on biomass and yield (Lawson 2014). Even in some horticultural leaf species, such as Chinese cabbage, the application of more than 25 mg kg soil^{-1} reduces the weight of the plants (Hong et al. 2008). The absorption efficiency of iodine in plants begins to decrease when applying 55 mg I kg soil^{-1} (Weng et al. 2009). With higher concentrations of 70, 60, and 110 mg I kg soil^{-1}, intoxication occurs, showing slight lesions, prolongation of germination time, growth retardation, wilting, and yellowing of the leaves in cucumber, eggplant, and radish. On the other hand, when using 140, 150, and 180 mg I kg soil^{-1} in the same crop species, the plants showed extensive foliar

Table 4.4 Some biofortification studies with iodine in different crop species

Crop	Chemical form	Concentration applied	Application	Results	Author
Celery	KI, KIO_3	0.05–10.0 mg L^{-1}	Nutrient solution	The stems of celery constituted an effective and stable store of iodine	Li et al. (2018)
Celery	KIO_3	0, 1.0, 5.0 mg I kg $soil^{-1}$	Soil	The concentration of iodine in the edible parts increased with the greater availability of iodine in the soil	Dai et al. (2004)
Rice	KIO_3	0.05% w/v	Foliar spray in the grain filling	Three applications of iodine substantially increased the concentration in the grain	Cakmak et al. (2017)
Eggplant	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Zucchini	KIO_3	10, 40, 80 μg L^{-1}	Applied to the soil through irrigation water	Higher accumulation of I when applied in 40 μg/L through fertigation	Ujowundu et al. (2010)
Pumpkin	KIO_3	10, 40, 80 μg L^{-1}	Applied to the soil through irrigation water	Higher accumulation of I when applied in 40 μg/L through fertigation	Ujowundu et al. (2010)
Onion	KIO_3	0, 1.0, 5.0 mg I kg $soil^{-1}$	Soil	The concentration of iodine in the edible parts increased with the greater availability of iodine in the soil	Dai et al. (2004)
Pea	KI, KIO_3	1000 mg I L^{-1}	Solution for seed treatment	Higher concentration of I in germinated treated seeds	Jerše et al. (2017)

(continued)

Table 4.4 (continued)

Crop	Chemical form	Concentration applied	Application	Results	Author
Coriander	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Turnip cabbage	KI, KIO_3	0, 1.0, 2.5, 7.5, and 15 kg I ha^{-1} to soil and 0.5 kg I ha^{-1} by foliar spray	Soil and foliar	The KIO^3 to the soil in 7.5 kg I ha^{-1} enabled the desired level of iodine in the plant (50–100 μg I 100 g FW^{-1}) The KI to the soil and the foliar application of KI and KIO_3 were not effective	Lawson et al. (2015)
Cabbage	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Chinese cabbage (pak choi)	KI, KIO_3	0.05–10.0 mg L^{-1}	Nutrient solution	The plant accumulated iodine in soluble forms (possibly in the apoplast and vacuole) proportionally to the amount applied in the nutrient solution	Li et al. (2018)
Chinese cabbage	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)

(continued)

Table 4.4 (continued)

Crop	Chemical form	Concentration applied	Application	Results	Author
Spinach	KI, KIO_3	1.1 mg I dm^{-3} soil	Pre-sowing application to soil and by fertigation	The application by the fertigation was more effective than the pre-sowing application in the soil	Smoleń et al. (2016a)
Spinach	KIO_3	0.0004% I in irrigation water	Fertigation combined with the application of humic and fulvic acids	The combination of KIO_3 with humic substances increased the concentration of iodine in plants	Smoleń et al. (2017)
Spinach	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Spinach	KIO_3	0, 1.0, 5.0 mg I kg $soil^{-1}$	Soil	The concentration of iodine in the edible parts increased with the greater availability of iodine in the soil	Dai et al. (2004)
Spinach	KI, KIO3	Pre-sowing 1 mg I dm^{-3} soil. Fertigation 0.0004% (equivalent to 1.1 mg I dm^{-3} soil)	Pre-sowing application to soil and by fertigation	The application by the fertigation was more effective than the pre-sowing application in the soil	Smoleń and Sady (2012)
Ceylon spinach	KIO_3	10, 40, 80 μg L^{-1}	Applied to the soil through irrigation water	Higher accumulation of I when applied at 40 μg/L through fertigation	Ujowundu et al. (2010)

(continued)

Table 4.4 (continued)

Crop	Chemical form	Concentration applied	Application	Results	Author
Strawberry	KI y KIO_3	$I^- \leq 0.25$ mg L^{-1} $IO_3 \leq 0.50$ mg L^{-1}	Nutrient solution	Iodine improved the quality of the fruit, and biofortified fruits were obtained with 600–4000 μg kg^{-1} FW	Li et al. (2017b)
Mung bean	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Ipomoea aquatica	I^- IO_3^- CH_2ICOO^-	0–1.0 mg L^{-1}	Nutrient solution	Iodine in the plant increased proportionally to the concentration in the nutrient solution. The greatest absorption was $CH_2ICOO^- > I^- > IO_3^-$	Weng et al. (2008b)
Lettuce	KI	0.5, 1, 2.5, and 5 μM dm^{-3}	Nutrient solution	The concentration of 0.5 μM dm^{-3} was sufficient to effectively biofortify the seedlings	Krzepiłko et al. (2016)
Lettuce	KI, KIO_3	0.25 kg I ha^{-1}	Foliar	The iodine content increased in the edible organs with the application of KI and KIO_3 via foliar shortly before harvest	Lawson et al. (2016)

(continued)

Table 4.4 (continued)

Crop	Chemical form	Concentration applied	Application	Results	Author
Lettuce	KI, KIO_3	0, 1.0, 2.5, 7.5, and 15 kg I ha^{-1} to soil and 0.5 kg I ha^{-1} by foliar spray	Soil and foliar	The KIO_3 to the soil in 7.5 kg I ha^{-1} and the foliar application achieved the desired level of iodine in the plant (50–100 μg I 100 g FW^{-1}) The KI to the soil was not effective	Lawson et al. (2015)
Maize	KIO_3	0.05% w/v	Foliar spray in the grain filling	Three applications of iodine substantially increased the concentration in the grain	Cakmak et al. (2017)
Potherb mustard	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Cucumber	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Pepper	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Pepper	KI	10–50 μM	Foliar spray	The concentration of iodine in the plants was proportional to that applied in the sprayed solution	Cortés-Flores et al. (2016)

(continued)

Table 4.4 (continued)

Crop	Chemical form	Concentration applied	Application	Results	Author
Pepper	KI	0.25–5.0 mg L^{-1} KI	Nutrient solution	The fruits reached 350–1350 μg kg^{-1} FW. Using 0.25–1 mg L^{-1} KI in the nutrient solution increased the fruit quality	Li et al. (2017a)
Cabbage	KIO_3	0.21, 22.7, and 0.59 kg ha^{-1}	Soil	The application to the soil increased the iodine content in the leaves	Mao et al. (2014)
Tomato	KI, KIO_3	1 mg dm^{-3}	Nutrient solution	In combination with salicylic acid, the content of I was increased in fruits	Smoleń et al. (2015)
Tomato	Algae and diatomite	12–150 mg I m^{-2}	Soil	The organic fertilizer increased the iodine concentration in the plants proportionally to the amount applied	Weng et al. (2013)
Tomato	KI, KIO_3	KI 1, 2, and 5 mM KIO_3 0.5, 1, and 2 mM	Nutrient solution, once a week on four occasions	The concentration of iodine in the biofortified fruits was proportional to the concentration in the nutrient solution	Kiferle et al. (2013)
Wheat	KIO_3	0.05% w/v	Foliar spray in the grain filling	Three applications of iodine substantially increased the concentration in the grain	Cakmak et al. (2017)

(continued)

Table 4.4 (continued)

Crop	Chemical form	Concentration applied	Application	Results	Author
Carrot	KIO_3	0, 1.0, 5.0 mg I kg $soil^{-1}$	Soil	The concentration of iodine in the edible parts increased with the greater availability of iodine in the soil	Dai et al. (2004)
Carrot	KI, KIO_3	5 kg I ha^{-1}	Soil	Fertilizing with KI and KIO_3 (5 kg I ha^{-1}) in combination with Na_2SeO_3 (1 kg ha^{-1}) produced carrots with 100% of the recommended daily intake of I per 100 g of FW	Smoleń et al. (2016b)
Polignano carrot	KIO_3	50 and 500 mg L^{-1} foliar spray and 50 mg L^{-1} in nutrient solution	Nutrient solution and foliar spray	The biofortification was effective applying 50 mg L^{-1} KIO_3 via foliar in the open field and the greenhouse. When I was applied in the nutrient solution, high iodine levels were reached for human consumption	Signore et al. (2018)
Water spinach (*Ipomoea aquatica*)	KIO_3	0, 1.0, 5.0 mg I kg $soil^{-1}$	Soil	The concentration of iodine in the edible parts increased with the greater availability of iodine in the soil	Dai et al. (2004)

The data shown indicate the plant species where various forms of application of iodine in different concentrations were applied, resulting in the biofortification of the edible parts

lesions and even death in the seedling stage (Weng et al. 2008c). For plants grown in soil with fertigation, biofortification with iodine has also been suggested (Dai et al. 2004; Ujowundu et al. 2010), combining the iodine with the application of humic substances or organic acids (Smoleń et al. 2015; 2016a).

In soilless crops, in leafy vegetables such as Chinese cabbage, spinach, and lettuce, the application of iodine in a concentration of 10^{-6} M (0.17 mg L^{-1} KI, 0.21 mg L^{-1} KIO_3) produces an increase in biomass (Borst Pauwels 1961; Whitehead 1973; Weng et al. 2003; Zhu et al. 2003; Dai et al. 2004; Weng et al. 2008b; Blasco et al. 2013). The iodine concentration to obtain an adequate result as biofortification can rise to 10^{-5} M (equivalent to 1.7 mg L^{-1} KI or 2.1 mg L^{-1} KIO_3) (Weng et al. 2008b). In strawberry plants, the nutrient solution with I^- in 0.25 mg L^{-1} (1.97×10^{-6} M) and the IO_3^- in 0.50 mg L^{-1} (2.86×10^{-6} M) were effective in increasing the biomass and biofortifying the fruits with iodine (Li et al. 2017b). As the amount of iodine in the nutrient solution increases, symptoms of intoxication develop, as in rice where an amount greater than 100 μM (16 mg L^{-1} KI, 21.4 mg L^{-1} KIO_3) in the nutrient solution produces adverse effects on biomass (Mackowiak and Grossl 1999; Singh et al. 2012). In lettuce, the same unfavorable outcome appears with 40 μM (6.6 mg L^{-1} KI, 8.6 mg L^{-1} KIO_3) in lettuce (Blasco et al. 2008) (Table 4.3). For fruit vegetables such as tomatoes, up to 4 mg L^{-1} (3.2×10^{-5} M) can be applied without affecting the biomass of the plants (Hageman et al. 1942). The use of iodine can focus on particular growth stages, as described by Kiferle et al. (2013) who applied high concentrations of iodine eight times once a week, starting with the fruit set of the first cluster. The authors used 1–5 mM of KI (166–830 mg L^{-1}) and 0.5–2 mM of KIO_3 (107–428 mg L^{-1}) in the nutrient solution, obtaining fruits biofortified with 10 mg of iodine per kg of fresh weight, with low impact due to phytotoxicity in plants. In other species where reserve organs such as onions are harvested, no effect of iodine on biomass was observed (Dai et al. 2004). However, a decrease in biomass has been reported when applying iodine to potatoes and tomatoes (Caffagni et al. 2011), carrots (Smoleń et al. 2014b), and nopal (García-Osuna et al. 2014).

Iodine interaction with other elements. From a nutritional perspective, a biofortification program with iodine should ideally occur without restrictions on other essential mineral elements (White and Broadley 2009). In lettuce, it was verified that the application to the soil of KI (0.5–2.0 kg ha^{-1}) and the foliar spraying of KIO_3 (0.02–2 kg ha^{-1}) did not change in a significant way the mineral composition of the lettuce, including elements such as Na, Al, Cd, and Pb (Smoleń et al. 2011). In experiments of biofortification with iodine in tomato, a positive correlation was found between the concentration of iodine and the concentration of Cu and Mn in the leaves (Hageman et al. 1942). In the lettuce, applying KI to the soil and KIO_3 by foliar spray, the same positive effect was found on the Mn but not on the Cu (Smoleń et al. 2011). (Smoleń et al. 2014a) reported that the joint application of KIO_3 and SeO_4^{2-} in hydroponic lettuce plants did not modify the biomass or mineral composition in lettuce, observing a synergy that resulted in higher absorption of both elements in the leaves. On the other hand, (Mao et al. 2014) when jointly applying Zn, Se, and I demonstrated the biofortification for the three elements in cabbage. (Hageman et al. 1942) suggested that the modification in mineral composition that occurs in plants

when applying iodine is possibly related to a redox phenomenon, since the oxidation of I^- to I_2 provides a reducing potential of −0.535 V. Similarly, the IO_3^- through the induction of the reductase activity in the root (Kato et al. 2013) could generate a similar effect. This redox effect of iodine is expected to have a higher magnitude in an edaphic system (with more interacting components) compared to a soilless production system (Jones 1998). The difference in the number of interactions between the components of each system could partially explain the diversity of results observed with the use of iodine in crops.

Biofortification and antioxidants in plants. In addition to the biofortification potential, exogenous applications of iodine have an impact on the total antioxidants and tolerance to stress in plants. These responses, except in a few species, have been little studied and are considered as an advantage that can be used to promote the use of iodine among agricultural producers (Medrano-Macías et al. 2016a). Iodine applications at adequate concentrations result in a positive impact on the antioxidant potential.

As for the responses of biomass and yield, a great variability occurs depending on the plant species and the growing conditions. In the tomato, it was reported that applying 7.88 μM IO_3^- increased the content of ascorbic acid and total phenolic compounds (Smoleń et al. 2015). In greenhouse tomato seedlings, the KI applied by foliar spray significantly increased the concentration of ascorbate and glutathione, without changing the enzymatic activity of catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX), but decreasing that of superoxide dismutase (SOD) (Medrano-Macías et al. 2016b). On the other hand, in tomato grown in sand, the I^- concentration of 4 mg L^{-1} (3.2×10^{-5} M) did not modify the biomass compared to the control, but the concentration of ascorbic acid in the foliage of the plants decreased (Hageman et al. 1942). In *Ipomoea aquatica,* the I^- induced a higher amount of ascorbic acid, while the IO_3^- and the iodoacetic acid (CH_2ICOO^-) had the opposite effect (Weng et al. 2008b). In soybean grown in pots with soil, KIO_3 with concentrations of 20, 40, and 80 μM increased the enzymatic activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Gupta et al. 2015).

In hydroponic lettuce the application of KI resulted in more significant accumulation of phenols, ascorbic acid, and an increase in antioxidant potential (Blasco et al. 2008), while the application of KI (20, 40, and 80 μM) and KIO_3 (20 μM) increased the concentration of ascorbic acid and the enzymatic activity of CAT, decreasing the concentration of glutathione (GSH) and the activity of SOD. As for the APX enzyme, the activity in lettuce was increased more efficiently by KIO_3 compared to KI (Blasco et al. 2011). The positive effect on enzymatic antioxidants such as superoxide dismutase (SOD) and ascorbate peroxidase (APX), as well as non-enzymatic antioxidants such as GSH, and ascorbic acid (AA) was presented with <40 μM of KIO_3 (Leyva et al. 2011). On the other hand, Blasco et al. (2013) evidenced the increase in the antioxidant response and higher concentration of total phenolic compounds by applying KIO_3 in concentrations of 20 and 40 μM. In nopal cultivation, (García-Osuna et al. 2014) observed the higher amount of ascorbic acid in plants grown in soil under plastic tunnels when applying 10^{-4} M of KIO_3 and KI by fertigation. In pea seeds, the simultaneous application of I and Se allowed to obtain biofortified seeds with

both elements, without modifying the concentration of glutathione, but decreasing the anthocyanins with the specific combination of I^- and SeO_4^{2-}, without presenting this adverse effect with the IO_3^- (Jerše et al. 2018).

4.6 Research Needs Regarding Iodine Biofortification

The information presented lists a large number of achievements in recent decades about the biofortification of crops with iodine. However, according to the author's criteria, a series of questions deserve attention:

1. The factors that promote the bioavailability of iodine in the soil are relatively well described. However, it is necessary to carry out more research to validate in different regions and climates, types of soil, and plant species the agronomic management directed to the biofortification of the crops with iodine. In fact, all the studies are directed toward the exogenous application of iodine, in different concentrations, chemical species, substrates, and plant species. No information was found about how agronomic management of soil, substrate, and plants or environmental conditions in the case of soilless production systems modify the concentration of iodine in crops, whether this occurs under deficit conditions of bioavailable iodine or with adequate concentrations of the element in soil or soil pore water.
2. The mobility of iodine through vascular systems is a subject that requires a lot of work and is essential to understand the accumulation of iodine in fruits and grains. It is necessary to understand better the connection between the microclimate of plants and transport through the xylem, as well as the external and internal factors that regulate the flow of iodine through the phloem.
3. It is necessary to verify the feasibility of biofortification with iodine in more crop species; almost all the research effort has been directed to 20–25 plant species. Those that accumulate significant reserves of carbohydrates and lipids in edible organs, especially if they are roots, stems, or leaves, can be suitable candidates for biofortification when the iodine is obtained from the soil. In the case of foliar spraying applications, potentially all plants with edible aboveground structures would be used as sources of iodine.
4. The issue of iodine stability once it is found in plant structures can be equally valuable. The factors that regulate its permanence or volatilization in the edible organs are little understood. A related issue would be the balance between conservation and volatilization after harvest and during storage, as well as the impact of the final food preparation processes.
5. Further study is required about organic sources of iodine. Firstly, macroalgae and marine and freshwater microalgae as a source of iodine-rich biomass, both for direct consumption and for the preparation of composts enriched with iodine. Second, biopolymers, minerals, and nanomaterials that form complexes with

iodine or absorb it in their polymeric networks or porous structures, serving as a source of slow release of the element and decreasing the volatilization rate of the same.

6. Regarding the interaction of iodine with other mineral elements, the available information seems to indicate that iodine does not induce significant restrictions on other mineral elements. However, a topic that has not been addressed with adequate extension is the simultaneous biofortification with I–Fe–Zn, I–Se, and even with I–Se–Si.
7. The facet of the use of iodine as an antioxidant to increase tolerance to stress is practically unexplored. The description of the impact of iodine for different kinds of biotic or abiotic stress could be crucial to increase acceptance of iodine use among agricultural producers.
8. Some basic knowledge may be useful. Examples are the impact that iodine has on transcriptomes or proteomes in plants, on metabolic efficiency (such as photosynthesis, respiration, metabolism of other mineral elements, among others), as well as on the microbiome of plants. These, among other topics, are very little studied and more understanding will undoubtedly allow us to understand and expand the uses of iodine in agriculture.

4.7 Concluding Remarks

The use of table salt iodization has solved many problems of iodine deficiency among the population. The biofortification of crop plants is a set of techniques that complement the iodization of salt and allow obtaining plant foods with concentrations of the element that partially or entirely covering the daily requirements of iodine intake. The concentration of iodine in food depends primarily on the ability of plants to absorb it and accumulate it and on the capacity of soil and water to provide it in bioavailable forms for plants. In many soils, the low concentration of bioavailable iodine is the result of intrinsic geological factors, but in other cases, it seems to be the result of edaphic fixation. These edaphic factors that modify the bioavailability of iodine in the soil are well known, pH and ORP, organic matter, minerals of colloids, and microbial action. Although almost all these edaphic factors form, or can be part of routine agronomic management, little is known about their appropriate combinations to increase the bioavailability of iodine that is fixed in agricultural soils, although it is possible that they are analogous to those needed to improve the bioavailability of other elements such as P and Si. In fact, the total experimental reports on biofortification refer to the contribution of exogenous iodine in mainly inorganic forms (KI and KIO_3) and some organic (kelp and iodinated organic acids) both in soil crops and on substrates other than soil or hydroponics. The ideal situation would be for exogenous applications to be used mostly for crops in soilless systems, whereas for the crops in soil, the exogenous applications were a complement to agronomic management aimed to promote the bioavailability of the iodine in the soil solution.

As iodine is not an element qualified as essential for plants, the extension of its use among agricultural producers will not be simple, unless it ensures some utilitarian facet of iodine, which could be its capacity to promote antioxidants and tolerance to stress.

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Chapter 5
Biofortification of Maize for Protein Quality and Provitamin-A Content

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Abstract Protein and micronutrient deficiency have emerged as the major public health problems in under-developed as well as developing countries. Of the several approaches used to alleviate malnutrition, biofortification has turned out to be the most effective and sustainable approach for providing micronutrients in natural forms. Marker-assisted selection (MAS) is an effective strategy to introgress trait(s) especially controlled by recessive gene(s). Maize is an important cereal extensively used as food and feed around the world. The nutritive value of maize, however, remains relatively poor on account of low quality of protein and lower level of micronutrients like provitamin-A. Research initiatives during the past three decades have culminated in the development of quality protein maize (QPM) that possesses nearly double the quantity of lysine and tryptophan, which helps in enhancing the biological value of QPM protein as compared to normal maize. QPM although contains better quality of protein; yet the quantity of provitamin-A is very low (<2 ppm). The current chapter presents an overview of the research work undertaken to enhance the quality of protein in maize grains on the one hand and the level of provitamin-A on the other. Two genes viz. *lycopene ε-cyclase* (*lcyE*) and *β-carotene hydroxylase1* (*crtRB1*) play a significant role in enhancing provitamin-A. Favourable alleles of *crtRB1* and *lcyE* gene(s) were introgressed in commercial hybrids using MAS. The resultant hybrids were found to contain 4.5-fold more provitamin-A as compared to original hybrids. Stability of provitamin-A in QPM as well as normal maize and possible impact of these multi-nutrient hybrids in reducing protein and vitamin-A deficiency have also been discussed.

Keywords Biofortification · Marker-assisted selection · Maize · Quality protein maize · Provitamin-A

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5.1 Introduction

Biofortification, known to enhance micronutrients in the edible part of the plants through breeding approaches, has turned out to be a sustainable, economic and robust way for supplying nutrients to human being (Bouis 2018). Staple food crops like wheat, rice, maize, millets and legumes are the major sources of food, energy, various minerals and vitamins required for growth and development (Neeraja et al. 2017). Cereals are the major source of carbohydrate and are moderate in protein quantity as well as quality. Some of the cereals, however, possess poor quality of proteins. Malnutrition caused by consumption of unbalanced food has emerged as a major health problem worldwide. Recent data indicate that around 2 billion people suffer globally from malnutrition, and of this, nearly 815 million are undernourished (Global Nutrition Report 2017). Of the 667 million children under the age of five, 155 million are stunted and 52 million do not weigh enough for their height. An estimated 45% of death of children under the age of five is linked to malnutrition (Black et al. 2013). Malnutrition also contributes to loss in annual gross domestic product (GDP) to an extent of 11% in Asia and Africa.

Alleviating malnutrition has been identified as the key step towards achieving sustainable development goals (SDGs) (Global Nutrition Report 2017). Biofortification of staple food crops has been identified as one of the most promising approaches to alleviate malnutrition. Among cereals, maize has attracted worldwide attention as staple food and feed, besides serving as raw material for an array of industrial products (Hossain et al. 2018). Along with rice and wheat, maize is a source of up to 30% of the calories for more than 4.5 billion people especially among developing countries (Shiferaw et al. 2011). Globally, more than a billion tonnes of maize grains were produced from 180 million hectares of land distributed among as many as 165 countries (Foreign Agricultural Service/USDA 2017). Maize grain is moderately rich in protein content but poor in the quality of protein on account of deficiency of two essential amino acids viz. lysine and tryptophan (Zunjare et al. 2018a; Sarika et al. 2018a). Intake of food deficient in essential amino acids results in increased susceptibility to diseases, decreased blood constituents and eventually retarded mental and physical development of children (Galili and Amir 2013). Among various micronutrient deficiencies, protein–energy malnutrition (PEM) also known as protein–energy undernutrition (PEU) causes highest number of death worldwide (Bain et al. 2013). Maize is also known to be deficient in provitamin-A (proA) and the low content present in endosperm cannot meet the daily requirement of human being (Muthusamy et al. 2014; Gupta et al. 2015). ProA is required for proper cell growth, eye vision and normal functioning of immune and reproductive system of human body (Sommer and West 1996). Around 4.4 million preschool-age children and 20 million pregnant women (one-third are clinically night blind) suffer from visible eye damage and night blindness due to vitamin-A deficiency (VAD). Recent study conducted by Skjaerven et al. (2018) revealed that micronutrient deficiency in parents affects the liver function of progeny. Since these micronutrients cannot be synthesised in human body, they need to be consumed through diet rich in micronutrients

(Tanumihardjo 2011). The recommended daily allowance of lysine is 30 mg/kg body weight for adults, while it is 35 mg/kg body weight for children. As regards tryptophan, the daily requirements are 4 and 4.8 mg/kg body weight/day in adults and children, respectively (WHO/FAO/UNU 2007). Similarly, World Health Organisation (WHO) has recommended an average proA of 500 RE (retinol equivalent) for adults and 250 RE for children per day (Bouis and Welch 2010). HarvestPlus, an international organization for development of nutrition-rich crops for human nutrition, has fixed a target of 15 ppm of proA in maize to meet the daily requirement (Andersson et al. 2017).

To combat protein–energy imbalance, plant breeders have developed quality protein maize (QPM) genotypes by using recessive *opaque2* (*o2*) allele (Mertz et al. 1964) in conjunction with endosperm modifiers at CIMMYT, Mexico (Vasal et al. 1980; Bjarnason and Vasal 1992). Natural variants of *β-carotene hydroxyalse1* (*crtRB1*) and *lycopene-ε-cyclase* (*lcyE*) have been used to develop proA-rich maize lines in various countries (Dhliwayo et al. 2014; Muthusamy et al. 2014; Simpungwe et al. 2017; Zunjare et al. 2017, 2018a). Here, we present an overview of the development, dissemination, impact and challenges in adoption of the of biofortified maize cultivars.

5.2 Biofortification for Sustainable Supply of Micronutrients Through Diet

This approach has become very popular among plant scientists for development of nutritionally enhanced food crops such as wheat, rice, maize, pearl millet, beans, sweet potato, etc. (Giuliano 2014; Owens et al. 2014). Although food fortification and supplementation are some of the various means deployed for alleviating malnutrition, they are not sustainable in the long run, primarily due to lack of purchasing power of the poor and unorganised distribution system. The prevalence of poverty in developing countries, poor infrastructure, and lack of awareness are also the major constraints for their success. Though dietary diversification holds promise, availability of fruits and vegetables is limited to seasons; and the poor cannot afford it on regular basis, hence this strategy also becomes non-sustainable (Bouis and Welch 2010; Tanumihardjo 2011; Vignesh et al. 2012). Amongst all, biofortification is regarded as the most sustainable and cost-effective and robust method for amelioration of malnutrition (Gupta et al. 2015). Biofortification has been attempted in various ways (alone and in combination); the major ones in maize are presented as follows.

5.2.1 Single Biofortification

The term 'single biofortification' refers to enhancement of a single or a group of similar micronutrient(s) through breeding approach in crops. For instance, introgression of recessive allele of *o2* in presence of modifiers led to development of QPM cultivars rich in two essential amino acids viz. lysine and tryptophan. Likewise, quantity of proA has been enhanced in maize genotypes through introgression of favourable alleles of *crtRB1* and *lcyE* genes (Vignesh et al. 2012, 2013; Babu et al. 2013; Choudhary et al. 2014, 2015; Muthusamy et al. 2014, 2015a, b, c, 2016; Liu et al. 2015; Zunjare et al. 2017, 2018a, b, c). Both conventional breeding and molecular breeding approaches have been deployed for enriching the micronutrients to agronomically superior lines/hybrids of maize.

5.2.1.1 Development of Quality Protein Maize (QPM)

The *o2*-based QPM contains nearly double the amount of lysine and tryptophan in the endosperm than the normal maize. Such balanced combination of amino acids in the endosperm results in higher biological value thereby ensuring availability of better quality of protein than normal maize. The recessive allele of *o2* was first described by Jones and Singleton in 1920 (Emerson et al. 1935), but its nutritional significance was discovered by Mertz et al. (1964) at Purdue University, United States of America. α-zeins are the most abundant storage proteins in the maize endosperm but they are characteristically poor in lysine and tryptophan. The homozygous *o2* mutant causes reduction in production of zeins resulting in a corresponding increase in non-zein proteins, which contain higher levels of lysine and tryptophan (Gibbon et al. 2003). The *o2* gene, located on the short arm of chromosome 7, encodes a leucine zipper transcription factor that regulates expression of the 19- and 22-kDa α-zeins. Mutation at the *o2* locus encodes the defective regulatory element resulting in reduced transcription of the α-zein genes. The *o2* gene also regulates the lysine ketoglutarate reductase (LKR) gene which degrades the free lysine (Schmidt et al. 1990). The *o2* mutation produces a defective transcriptional factor resulting in reduced transcription of the LKR and thereby less degradation of free lysine (Brochetto-Braga et al. 1992). Further, *o2* is also generally accompanied with increased proteins relatively rich in lysine (Jia et al. 2013). In addition, QPM also balances leucine–isoleucine ratio for tryptophan liberation which enhances niacin biosynthesis and thereby combats pellagra disease.

Although the *o2* allele modified the amino acid pattern beneficially, yet; it possessed undesirable traits like low yield, soft and chalky endosperm, more susceptibility to diseases and insect–pests and higher storage losses. Since grain yield is a primary trait of interest, the soft endosperm-based *o2* cultivars were not accepted by the farmers. Later, breeders at International Maize and Wheat Improvement Center (CIMMYT), Mexico combined *o2* and endosperm modifiers, which resembled normal maize both in kernel phenotype and agronomic performance, and named

the lysine and tryptophan-rich genotype as QPM (Vasal et al. 1980). The breeding schemes followed and germplasm used in the development of QPM have been mentioned in several reviews published earlier (Bjarnason and Vasal 1992; Vasal 2000, 2001; Prasanna et al. 2001).

The genetic and molecular basis of modifier loci that affect both kernel modification and amino acid accumulation in endosperm of QPM genotypes assumes great significance. The factors responsible for the formation of vitreous and starchy endosperm in maize are poorly understood (Holding et al. 2008). The inheritance pattern of modifiers is fairly complex and quantitative in nature with preponderance of additive gene effects, and occurrence of reciprocal cross-difference, dosage and xenia effects (Lopes and Larkins 1995; Bjarnason and Vasal 1992). However, molecular dissection of the trait showed increased levels of 27-kDa γ-zeins as likely candidate to impart hardness in endosperm, as the *o2*-modified (QPM) grains have approximately double the amount of γ-zein in the endosperm relative to the soft *o2* mutants (Wu et al. 2010). For selection of these modifiers, light box test is commonly deployed by the breeders in QPM breeding programme (Hossain et al. 2007, 2008a, b; Vivek et al. 2008). Genetic analyses on QPM germplasm suggest involvement of two independent loci controlling endosperm modification (Lopes and Larkins 1995). Bulked segregant analysis (BSA) of segregating populations also reveals two chromosomal regions on the long arm of chromosome 7 associated with the endosperm modification (Lopes et al. 1995). It has been demonstrated that γ-zeins and their interaction with starch granules are also involved in the modification of *o2* endosperm (Gibbon et al. 2003; Wu et al. 2010). The proteins of modifier genes can interact with γ-zein RNA and enhance their transport from nucleus and thus increase their stability (Dannenhoffer et al. 1995). Besides, γ-zein being enriched with cysteine residues, help in formation of disulphide bond between the protein bodies, thereby compacting the endosperm (Burnett and Larkins 1999). Holding et al. (2008) identified seven quantitative trait loci (QTLs) by using recombinant inbred line (RIL) population and characterised 24 candidates genes that are differentially regulated in QPM genotype, compared to the *o2* starchy mutant. Microarray hybridisation showed that some of the modifiers are associated with ethylene and ABA signalling and suggest a potential linkage of *o2* endosperm modification with programmed cell death (Holding et al. 2011). Liu et al. (2016) recently identified a QTL (*qγ27*) affecting expression of 27-kDa γ-zein, and it was mapped on chromosome 7 near the 27-kDa γ-zein locus. The QTL, *qγ27* resulted from 15.26-kb duplication at the 27-kDa γ-zein locus, increases the level of gene expression.

QPM also comprised of a distinct set of amino acid modifier genes which affect the relative levels of lysine and tryptophan content in the grain endosperm as the amount of essential amino acids in QPM genotypes varies quite extensively, indicating the role of modifiers in regulating amino acid biosynthesis (Pandey et al. 2015). Aspartate pathway directs lysine synthesis and is feedback-regulated by its end products. Aspartate kinase 2 (*Ask2)* gene located on long arm of chromosome 2 encodes aspartate kinase that catalyses the conversion of aspartate to β-aspartyl phosphate, and sensitive to lysine inhibition. However, natural mutant forms of *ask2* are feedback insensitive and aid in higher amounts of lysine, methionine and threonine

accumulation (Wang et al. 2007). Habben et al. (1995) demonstrated that elongation factor 1α (EF-lα) is over-expressed in *o2* endosperm compared to its wild types, and it is a significantly correlated with total lysine content of the endosperm. EF-1α protein is generally correlated with cytoskeleton network, mitotic apparatus, microtubule and many cellular processes (Lopez-Valenzuela et al. 2004). Genetic studies revealed two QTLs that account ~25% of the phenotypic variance. One locus on chromosome 4S is coincident with zein gene and hence allelic variation of α-zein may contribute to difference in EF-Iα content among parents by increasing the surface area protein bodies in the endosperm creating more extensive network of cytoskeleton (Wang et al. 2001). Wang and Larkins (2001), Holding et al. (2008), Gutierrez-Rojas et al. (2010), Pineda-Hidalgo et al. (2011), Lebaka et al. (2013) and Babu et al. (2015) have reported several modifier loci affecting the accumulation of lysine and tryptophan.

QPM Through Conventional Plant Breeding

Efforts were made on large-scale to develop QPM germplasm in tropical-, subtropical- and highland-genetic background with different maturity groups. Considering the complexity of QPM breeding system, an innovative breeding scheme, named as 'modified backcrossing-cum-recurrent selection', was used (Vasal et al. 1980, 1984). Many advanced maize populations were successfully converted to QPM versions through this procedure at CIMMYT, Mexico which is widely used in the development of QPM cultivars in several countries including Brazil, China, Ghana, India and several Latin American countries (Vasal 2001; Gupta et al. 2009; Tandzi et al. 2017). Some of the very popular QPM cultivars that merit mention are Obatampa (Ghana), AMH760Q (Ethiopia), Longe-5 (Sudan), Yunrui-1 (China), Poshilo Makai-1 (Nepal) and Chaskarpa (Bhutan). In India, during 1997, a nutritionally superior QPM composite with vitreous grain texture, 'Shakti-1', was released. Since 1998, intensive efforts in different breeding centres of India resulted in the release of QPM hybrids like Shaktiman-1, Shaktiman-2, Shaktiman-3, Shaktiman-4, Shaktiman-5, HQPM-1, HQPM-4, HQPM-5, HQPM-7 and Pratap QPM Hybrid-1. Bangladesh has also released QPM hybrid, BARI Hybrid Maize-5. In Pakistan, QPM hybrids, QPHM200 and QPHM300 were released recently in 2017. QPM cultivation and grain yield potential are interchangeable with normal maize (Ekpa et al. 2018; Hossain et al. 2018).

QPM Through Molecular Marker-Assisted Backcross Breeding (MABB)

The recent advances in plant biotechnology including discovery of molecular markers have accelerated introgression of the target gene(s) in high-yielding varieties/lines (Varshney et al. 2012). Conventional breeding approach to develop QPM genotypes is tedious and time-consuming (Gupta et al. 2013); consequently, introgression of *o2* through conventional backcrossing becomes demand due to several reasons viz. (i) inability to identify *o2* recessive allele in each backcross generations, (ii) requirement

of about six generations of backcrossing to recover satisfactory levels of recurrent parent genome and (iii) cumbersome biochemical tests of lysine and tryptophan levels in the selected materials in each generation. These steps require enormous labour, time and material resources. Marker-assisted backcross breeding (MABB), on the contrary, offers tremendous potential to improve the efficiency and accuracy of selection (Collard et al. 2005; Gupta et al. 2013). It involves two major strategies: (i) foreground selection: selection of the targeted gene through molecular marker(s) and (ii) background selection: recovery of the recurrent parent genome (RPG) using markers distributed throughout the genome (Hospital et al. 1992). Foreground selection precisely identifies the gene of interest, while background selection expedites the rate of genetic gain/recovery of RPG (Hospital et al. 1992; Babu et al. 2005; Gupta et al. 2013; Hossain et al. 2013). The molecular cloning of *O2* gene and its further characterisation (Schmidt et al. 1987; Motto et al. 1988), followed by discovery of three simple sequence repeats (SSRs) (*phi057*, *phi112* and *umc1066*) within the gene (Lin et al. 1997), led to the easy identification of the recessive *o2* allele among segregating generations (Babu et al. 2005; Gupta et al. 2013). These *o2*-based SSRs were further used in MABB programme that aimed to convert non-QPM lines into their QPM versions (Babu et al. 2005). ICAR-Vivekananda Parvatiya Krishi Anusadhan Sansthan (Vivekananda Institute of Hill Agriculture), Almora in India, developed 'Vivek QPM9', a QPM hybrid through marker-assisted selection (MAS) and the hybrid was released for commercial cultivation during 2008. The team at Almora employed phenotypic selection for endosperm modifiers, while MAS for *o2* allele and genome-wide SSRs in the parental lines of Vivek Hybrid 9 (CM145 and CM212) (Gupta et al. 2013). The QPM version, Vivek QPM-9 possesses 41% more tryptophan and 30% more lysine, with similar grain yield potential compared to original hybrid. Vivek QPM-9 earned the distinction of being the first MAS-based maize cultivar released for commercial cultivation in India (Gupta et al. 2013). Furthermore, three essentially derived varieties (EDVs) viz. 'Pusa HM4 Improved', 'Pusa HM8 improved' and 'Pusa HM9 Improved' possessing high lysine and tryptophan were developed through MAS at the ICAR-Indian Agricultural Research Institute, New Delhi and these hybrids were released for commercial cultivation during 2017 (Fig. 5.1). 'Pusa HM4 Improved' possesses high lysine (3.62%) and tryptophan (0.91%) in protein with an average grain yield of 6.4 t/ha. 'Pusa HM8 Improved' and 'Pusa HM9 Improved' possessed 4.18 and 2.97% of lysine, and 1.06 and 0.68% of tryptophan, with grain yield of 6.3 and 5.2 t/ha, respectively (Yadava et al. 2017; Hossain et al. 2018). In addition, several researchers worldwide have used molecular markers to introgress *o2* allele into normal inbreds (Manna et al. 2005; Danson et al. 2006; Magulama and Sales 2009; Jompuk et al. 2011; Kostadinovic et al. 2016).

Enhancement in the quantity of lysine and tryptophan helps in doubling the biological value of the maize protein in QPM. Therefore, in order to further increase the quantity of these two essential amino acids, Sarika et al. (2017) deployed another mutant *opaque16* (*o16*) and reported distinct advantages of using *o16* and *o2* alleles together. Linked SSRs, *umc1141* and *umc1149*, were successfully utilised for selection of *o16* (Yang et al. 2005, 2013; Zhang et al. 2010, 2013). As this mutant does not influence the physio-chemical properties of grains, utilisation of *o16* offers potential

of further increasing lysine and tryptophan in QPM lines/hybrids (Sarika et al. 2018a). The marker-assisted pyramiding of *o2* and *o16* revealed additional increase in lysine and tryptophan in *o2o2/o16o16* genotypes than only *o2o2*-based genotypes (Sarika et al. 2018b). Four popular commercial *o2*-based QPM hybrids, HQPM1, HQPM4, HQPM5 and HQPM7, were pyramided with *o16* allele. The reconstituted hybrids possess an average of 0.13% tryptophan and 0.50% lysine compared to 0.08 and 0.37% in original hybrids, with an average enhancement was 60 and 49%, respectively (Fig. 5.2) and similar yield potential too.

QPM Through Transgenic Approach

Transgenics has also been explored in enhancing protein quality in maize. Unger et al. (1993) created two mutants of wild type *O2* allele by deleting the basic domain and first 175 N-terminal residues. The mutants when co-expressed with *O2* inhib-

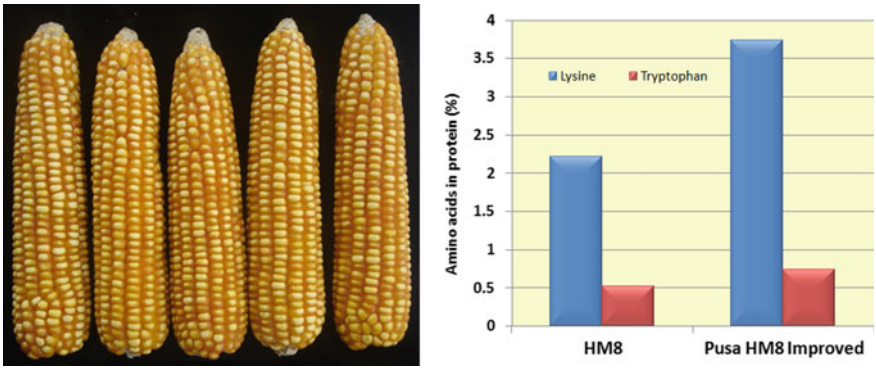

Fig. 5.1 Grain and ear characteristics of MAS-derived QPM hybrid 'Pusa HM8 Improved' and comparison of essential amino acids with original hybrid HM8

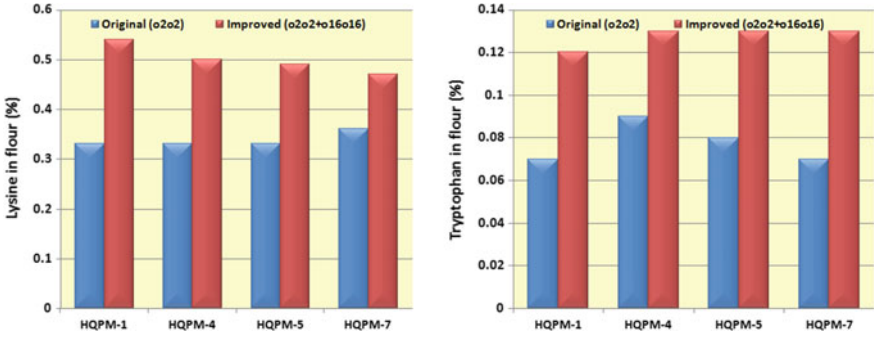

Fig. 5.2 Amino acid profiles in *o2o2* and *o2o2/o16o16*-based hybrids

ited expression of 22-kDa α-zein by nearly ten-fold in suspension cells of maize endosperm. The reduction in 22-kDa α-zein caused concurrent increase in non-zein proteins that are rich in lysine and tryptophan. Further, Segal et al. (2003) developed RNA interference (RNAi) constructs derived from a 22-kDa α-zein and produced a dominant opaque phenotype. Wu and Messing (2011) directed an RNAi construct against both 22- and 19-kDa α-zeins, and transgenic plants showed significant reduction in synthesis of zeins and recorded high-lysine concentration. These transgenic maize lines with great promise are; however, yet to be deployed for commercial production of maize hybrids with enhanced lysine and tryptophan.

5.2.1.2 Development of ProA-Rich Maize

Among the genes in the carotenoids biosynthesis, *lcyE* and *crtRB1* have been shown to predominantly regulate the accumulation of proA compounds and are the major target genes for the improvement of proA in the breeding programmes (Vallabhaneni et al. 2009; Babu et al. 2013; Vignesh et al. 2012, 2013; Muthusamy et al. 2015a, b, c, 2016; Zunjare et al. 2017, 2018a, b, c). Harjes et al. (2008) showed that a variation at the *lcyE* gene (bin 8.05) alters flux down α-carotene versus β-carotene branch explaining 58% of the variation in these branches and a three-fold difference in proA compounds. The *crtRB1* gene (bin 10.05) catalyses conversion of β-carotene into β-cryptoxanthin and further β-cryptoxanthin to zeaxanthin (Yan et al. 2010; Vignesh et al. 2013). The strong and statistically significant effect (2–10-fold) of favourable allele of *crtRB1* for enhanced β-carotene in maize is now very well established and has been used to develop proA-rich maize lines/hybrids (Azmach et al. 2013; Babu et al. 2013; Muthusamy et al. 2014; Liu et al. 2015; Menkir et al. 2017; Zunjare et al. 2017; 2018a). Positive effects of pyramiding of *crtRB1* and *lcyE* for proA enhancement were also shown by several researchers around the world (Babu et al. 2013; Azmach et al. 2013, 2018; Zunjare et al. 2017; Gebremeskel et al. 2017).

ProA-Rich Maize Through MABB

In proA biofortification programme, the quantification proA of samples using HPLC is not only tedious but expensive too (Pixley et al. 2013; Babu et al. 2013). Since MAS eliminates the need for phenotypic evaluation of large progenies, the introgression of favourable alleles of the two key genes viz. *lcyE* and *crtRB1*, imparting enhancement of proA, was employed. In the past, several proA-rich hybrids/open-pollinated varieties (OPVs) developed by CIMMYT, Mexico, were released in Malawi, Zambia and Zimbabwe. More than 15 proA-rich OPVs developed by International Institute of Tropical Agriculture (IITA), Ibandan, were also released in Nigeria, Ghana and DR Congo (www.harvestplus.org). Out these, three maize hybrids from Zambia (GV662A, GV664A and GV665A), two hybrids (Ife maize hyb-3 and Ife maize hyb-4) and two synthetics (Sammaz 38 and Sammaz 39) from Nigeria and one synthetic from Ghana

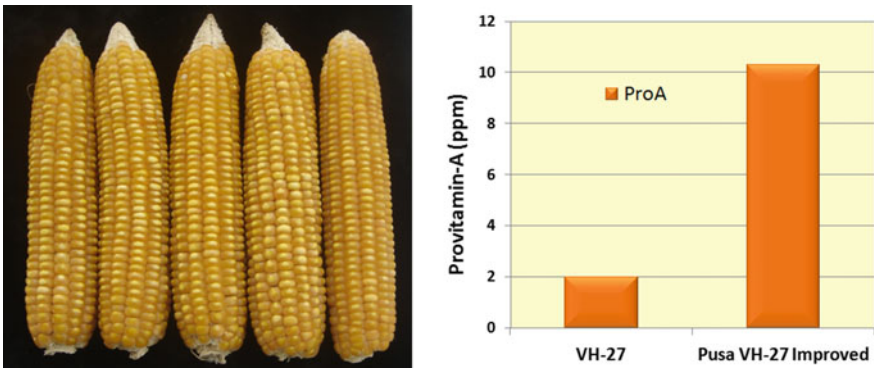

Fig. 5.3 Grain and ear characteristics of MAS-derived proA version of Vivek Hybrid-27, and comparison of proA (two months after storage) with original hybrid

(CSIR-CRI Honampa) possessing 6–8 ppm of proA are worth mentioning (Dhliwayo et al. 2014; Simpungwe et al. 2017). To date more than 40 proA maize varieties comprising of synthetics, single-cross hybrids, three-way hybrids have been released in many African countries such as DR Congo, Ghana, Malawi, Mali, Nigeria, Rwanda, Tanzania, Zambia and Zimbabwe (Andersson et al. 2017). Breeders at ICAR-IARI, New Delhi, have also introgressed the favourable allele of *crtRB1* gene from CIMMYT-HarvestPlus genotypes in the parental inbreds of popular maize hybrids viz. HM4, HM8 and Vivek Hybrid-27 using MABB approach (Muthusamy et al. 2014) (Fig. 5.3). The improved hybrids possessed higher proA as high as 21.7 ppm (in freshly harvested grains) with maximum change of 8.5-fold. Similar grain yield potential was observed in the improved hybrids compared to the original hybrids.

ProA-Rich Maize Through Transgenic Approach

Transgenic technology was also deployed to enrich the carotenoids in maize (Aluru et al. 2008; Zhu et al. 2008; Naqvi et al. 2009). Over-expression of *crtB* and *crtI* genes from bacteria (*Erwinia herbicola*) helped in increasing β-carotene content up to 10 ppm in Hi-II maize line (Aluru et al. 2008). This was followed by two reports on development of transgenic maize genotypes with ~60 ppm β-carotene using combination of five genes (*psy1*, *crtI*, *lycb*, *bch* and *crtW*) by Zhu et al. (2008) and Naqvi et al. (2009); however, the deployment of these lines for commercial cultivation/development of hybrids is yet to come.

5.2.2 *Double Biofortification*

The term 'double biofortification' refers to development of crop varieties enriched with the two diverse micronutrients by either sequential or simultaneous stacking of genes imparting higher micronutrient concentration. As for example, marker-assisted stacking of *crtRB1*, *lcyE* and *o2* for development of varieties rich in both essential amino acids and proA concentration (Muthusamy et al. 2014; Liu et al. 2015; Zunjare et al. 2018a). Both *crtRB1* and *lcyE* genes have contributed for enhanced proA, while *o2* allele enhanced the amount of essential amino acids viz. lysine and tryptophan in the same genetic background. This introgression of different favourable alleles of various genes in a single variety has become now a reality due to use of molecular markers that reduce the phenotypic screening of individuals drastically. Otherwise, to achieve double-biofortified crops, it would have taken enormous time and cost for phenotypic screening and thereby posing huge constraints.

5.2.2.1 Development of ProA-Rich QPM

Globally for the first time, a double-biofortified maize hybrid 'Pusa Vivek QPM 9 Improved' has been developed (in India), that possesses higher proA (8.15 ppm after two months of storage) and high tryptophan (0.74%) and lysine (2.67%) (Muthusamy et al. 2014; Yadava et al. 2017). The hybrid is released and notified for commercial cultivation in hill and peninsular India during 2017. The hybrid showed an average grain yield is 5.6–5.9 t/ha, with a potential grain yield of 8.0–9.4 t/ha in two diverse ecologies of the India. It was developed through introgression of *crtRB1* allele in *o2*-based hybrid, 'Vivek QPM9' at ICAR-IARI, New Delhi. Later, four QPM hybrids viz. HQPM1, HQPM4, HQPM5 and HQPM7 (popular maize hybrids recommended for commercial cultivation in India) were also pyramided with *crtRB1* and *lcyE* favourable alleles for elevating proA concentration in the QPM genetic background. The introgressed hybrids showed a mean of 4.5-fold increase in proA (range of 9.25–12.88 ppm) compared to original hybrids (2.14–2.48 ppm) after two months of storage. Essential amino acids, lysine (0.334%) and tryptophan (0.080%) of the improved hybrids were *at par* with the original QPM versions (lysine: 0.340% and tryptophan: 0.083%). In addition, the improved hybrids showed similar yield potential (6.3–8.5 t/ha) with their original versions (6.1–8.4 t/ha) when evaluated at multi-locations (Zunjare et al. 2018a). The proA improved versions of HQPM5 and HQPM7 hybrids (Fig. 5.4) have been identified for release in Annual Maize Workshop held at Assam Agricultural University, Jorhat, Assam in 5-7 April, 2019 (https://iimr.icar.gov.in). While, proA improved version of HQPM1 is under final year of testing in AICRP trial. In China, Liu et al. (2015) attempted improvement of proA in QPM inbreds by stacking *crtRB1* allele and *o2* in genetic background of two inbreds, CML161 and CML171. Enhancement of proA to 5.25 ppm from 1.60 ppm in CML161 and 8.14 ppm from 1.80 ppm in CML171 has been reported but their commercial release has not been reported so far in China or elsewhere.

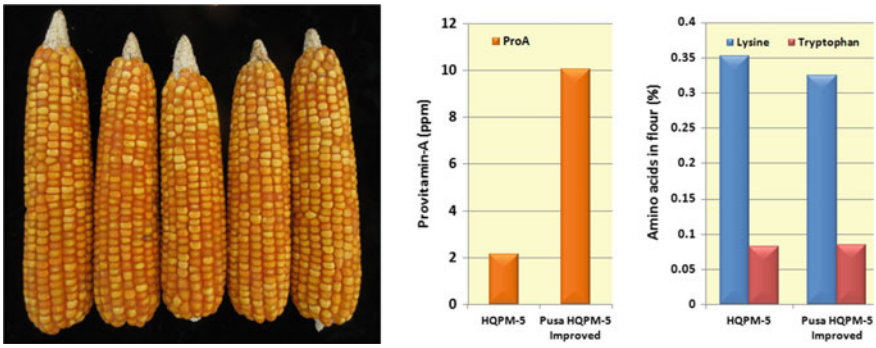

Fig. 5.4 Grain and ear characteristics of proA-rich MAS-derived version of QPM hybrid (HQPM-5), and comparison of proA (after two months of storage) and amino acids with original hybrid

Thus, the above reports show the success of biofortification in maize with either one, two or multiple nutrients, which will help in combating malnutrition in the countries where maize is used as staple cereal.

5.3 Nutritional Benefits of QPM

Meta-analysis of experiments conducted in different countries showed a strong implication about the nutritional benefits of QPM on gain of weight and height in infant and young children (Teklewold et al. 2015). The endosperm protein of QPM has been found to be equivalent to 90% of the milk protein (casein) as compared to the 40% in normal maize (Bressani 1994; Prasanna et al. 2001). The nutritional and biological superiority of QPM have been well established in rats, pigs, infants and small children as well as adults (Prasanna et al. 2001). In Guatemala, it was demonstrated that QPM with *o2* allele has 90% of the nutritive value of milk protein in young children. Children in Colombia suffering from 'Kwashiorkor', a severe protein deficiency disease, were brought back to normalcy on a diet containing QPM-based diets (Bressani 1994; Prasanna et al. 2001). Studies comprising of animal feeding trials also evaluated the nutritional value of QPM as animal feed over normal maize. Paes and Bicudo (1994) reported an increment of 50% in white and 40% in yellow cultivars in lysine, and corresponding 40 and 35% in tryptophan for QPM compared with the normal maize. Twelve per cent gain in rate of growth in weight and 9% increase in the rate of growth in height have been reported in infants and young children consuming QPM over the conventional maize (Gunaratna et al. 2010). The palatability and cooking quality of traditional food prepared from QPM are more acceptable due to its softness, perceived sweetness and longer shelf life in eastern African countries (Akalu et al. 2010). Tessema et al. (2016) studied the translating the impact of QPM

into improved nutritional status among Ethiopian children. Studies on rats found that the animals fed with QPM diet weighed more and were thicker, longer, denser and stronger than those fed on normal maize (Serna-Saldívar et al. 1992). Burgoon et al. (1992) found that pigs raised on QPM have twice the rate of weight gain. QPM in poultry diet also improves the growth performance of broilers and results in higher weight gain when replaced with normal maize (Nyanamba et al. 2003; Onimisi et al. 2008; Panda et al. 2014). The nutritional evaluation of QPM in feeding trials thus has proved its nutritional superiority over normal maize for human and livestock consumption (Nyakurwa et al. 2017; Tandzi et al. 2017).

5.4 Nutritional Benefits of ProA-Rich Maize

The importance of proA maize for health has been well established across the countries (Bouis and Saltzman 2017). Several studies on bioavailability of proA from biofortified maize clearly show that it is more efficient than the conversion ratio of 12:1 (into RE) as earlier proposed by the USA Institute of Medicine (2001). Estimates of 2.8:1 (Howe and Tanumihardjo 2006a, b), 3.2:1 (Muzhingi et al. 2011), and 6.5:1 (Li et al. 2010) for bioconversion has now been reported. Recently Dube et al. (2018) reported that consumption of 200 g of proA-rich maize would provide at least 50% of recommended diatary allowance, compared to 400 g as indicated by HarvestPlus programme (Bouis et al. 2011), thereby suggesting more efficient conversion ratio. An animal study on testing the bioavailability and an in vitro simulated digestion/Caco-2 cell study testing bioaccessibility of biofortified maize supports the findings of human studies in terms of efficient absorption (Howe and Tanumihardjo 2006b; Thakkar and Failla 2008). According to another study conducted in Zambia, the consumption of proA biofortified maize increased serum xanthophylls and ^{13}C-natural abundance of retinol in children (Sheftel et al. 2017). Data on intervention group in 679 Zambian children have also shown that consumption of β-carotene-rich maize significantly improved serum β-carotene concentrations (0.273 μmol/L) compared with traditional maize (0.147 μmol/L) (Palmer et al. 2018).

ProA maize has also emerged as an alternative to colour additives in poultry industry (Diaz-Gomez et al. 2017). Chickens fed with biofortified maize produced eggs rich in proA (Liu et al. 2012; Heying et al. 2014; Moreno et al. 2016; Sowa et al. 2017). Another study further reveals that chickens fed with proA biofortified maize had higher redness and yellowness and lower lightness in the meat and skin colour than the chickens fed with white maize (Odunitan-Wayas et al. 2016). Heying et al. (2014) showed that eating proA carotenoids daily at the time of gestation and lactation enhanced liver retinol status in piglets. Thus, both direct consumption through food and indirect consumption through poultry, proA-rich maize contributes significantly to nutritional security. Lividini and Fiedler (2015) demonstrated great promise of proA-rich maize as a highly cost-effective strategy for reducing malnutrition.

5.5 Scope and Challenges for Dissemination of Biofortified Maize

Thus, the deployment of popular maize hybrids enriched with micronutrients in high-yielding genetic background should prove beneficial for health and well-being of the people (Gupta et al. 2015). Furthermore, biofortified maize with enhanced lysine, tryptophan and proA has enormous potential to alleviate/reduce PEM and VAD either individually or simultaneously. Among various countries, Ghana cultivates QPM in 70,000 ha of land, while Mexico accounts for 2.5 million hectares of QPM (Nedi et al. 2016). However, despite great potential, area under QPM comprises only 1% of the total global area (Tandzi et al. 2017). Therefore, there exists huge scope for popularising QPM meeting protein requirement. The successful acceptance and consumption of biofortified maize cultivars depend on various factors like education of the household head, farmers' participation in demonstration trials, attendance to field days and numbers of livestock owned by the farmers (Gregory and Sewando 2013; Zuma et al. 2018). Further, two important behavioural barriers that exist between the development of biofortified maize and its impact on children's nutrition and health in practice are (i) the decision by households to adopt biofortified maize and (ii) the subsequent decision to allocate the improved maize to young children (Tessema et al. 2016). Lack of awareness on health benefits of biofortified maize is one of the major factors for its slow dissemination. In addition, the apprehension of low-yield potential of biofortified maize hybrids prevails in the mind of farmers and this needs proper counselling through intensive extension services. Dilution of nutritional quality by contamination of foreign pollen grains from neighbouring maize fields is yet another concern for the quality produce. The adoption of biofortified maize has also been limited due to lack of profitable markets for commercial producers, unwillingness among maize food processors in its marketing as a premium product, and absence of government incentive to encourage adoption by subsidising the price of seeds of biofortified cultivars. Inclusion of biofortified products in government sponsored health benefits programmes especially for children, pregnant women and elderly people would help in their quick dissemination. These factors present direct and indirect negative influence on popularity of biofortified maize. Hellin and Erenstein (2009) had identified (i) weak linkages between maize farmers and local poultry firms, (ii) limited access to improved technology and channels of information and other business services for small-scale maize and poultry producers and (iii) low prevalence of value chains with both growth and poverty reduction potential, as the three major challenges of the biofortified maize as poultry feed and maize-poultry value chains. Therefore, concerted efforts should be directed to create awareness among the growers, consumers and policy makers to enhance the area, consumption and popularity of the biofortified maize hybrids. The government of Ethiopia has set a target to have QPM varieties cultivated on 20% of the country's total maize area in the coming few years (Tessema et al. 2016). Addressing the above-mentioned interventions would pave way for the popularisation of QPM cultivars worldwide in general and south Asia in particular.

5.6 Conclusion and Way Forward

The world has witnessed an extraordinary impact of QPM hybrids in tackling the problem of PEM in many African countries. Furthermore, micronutrient (vitamin A, Fe and Zn) deficiency has turned out to be a major health problem in developing economies and normal maize contains less proA (<2.0 ppm) compared to target level proA (15.0 ppm) set by HarvestPlus. Fe and Zn are also low in maize grains. QPM hybrids, though rich in lysine and tryptophan, are devoid of sufficient proA in endosperm. Natural variant of *crtRB1* and *lcyE* has proved vital in improving QPM genotypes for enhancing concentration of proA. After the successful impact of QPM worldwide, proA-rich maize cultivars are currently being adopted by different stakeholders in African as well as Asian countries. Double-biofortified (proA + QPM) maize hybrids will have twin advantages of alleviating/reducing PEM and VAD, simultaneously. Further, the targeted incorporation of *VTE4* gene in QPM and proA-rich maize is underway to develop vitamin-E, proA, lysine and tryptophan-rich maize hybrids at ICAR-IARI, New Delhi, India. Enhancement of kernel iron and zinc through lowering phytic acid content also hold great significance. The multi-nutrient rich maize hybrids would go a long way in alleviating/reducing malnutrition through holistic approach.

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Chapter 6
Biofortification of Crops with Folates: From Plant Metabolism to Table

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Abstract Folates are micronutrients, also known as vitamin B9. Folate malnourishment is a global problem that affects human health from conception to old age. Folate nutrition has been ameliorated by mandatory flour fortification with folic acid in several countries; however, there still are populations with folate deficiency. Plants are the primary folate source in the human diet; thus, biofortifying crops with folates can be an alternate strategy to provide the vitamin to populations at risk. Plants synthesize folates in a complex, highly compartmentalized route and, as in other organisms; folates serve as cofactors of enzymes involved in the transfer of one-carbon (1C) groups, known as 1C metabolism. Proof-of-concept folate biofortification has been achieved in crops targeting different edible plant tissues: rice, maize, and common bean seeds, potato tubers, tomato fruit, and lettuce leaves. Engineering strategies included the overexpression of enzymes involved in the biosynthetic route and also protection of the molecule from degradation *in planta*, as folates are very labile compounds and food processing negatively affects their accumulation. Enhancing folate contents in food crops has required and also generated knowledge about the biochemistry of folate synthesis, transport, 1C metabolism, and stability within plant food matrices. In this chapter, we attempt to cover these aspects and also discuss the potential for developing folate biofortified crop varieties.

Keywords Folate · Biosynthesis · Degradation · Transport · Stability · Biofortification

6.1 Introduction

Folate is the general term for tetrahydrofolate (THF) and its derivatives. Folates are enzyme cofactors capable of receiving and donating one-carbon (1C) units; these reactions are part of the 1C metabolism present in all organisms. The folate molecule

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is composed of a pteridine ring attached to a *p*-aminobenzoate (PABA) moiety with one or several glutamates linked to it. The transferable 1C unit is attached to the N5 of the pteridine ring and/or N10 of the PABA structure at different oxidation levels (Fig. 6.1). Folates are produced by prokaryotes and some eukaryotes; animals do not have this biosynthetic capability and must consume folates as part of their diet. Also known as vitamin B9, these cofactors are essential micronutrients for human nutrition, and plants are the primary source. In human metabolism, 1C units transferred by folates are needed in several pathways: methionine, DNA, RNA synthesis, the methylation cycle, and NADPH production (Bekaert et al. 2008; Fan et al. 2014).

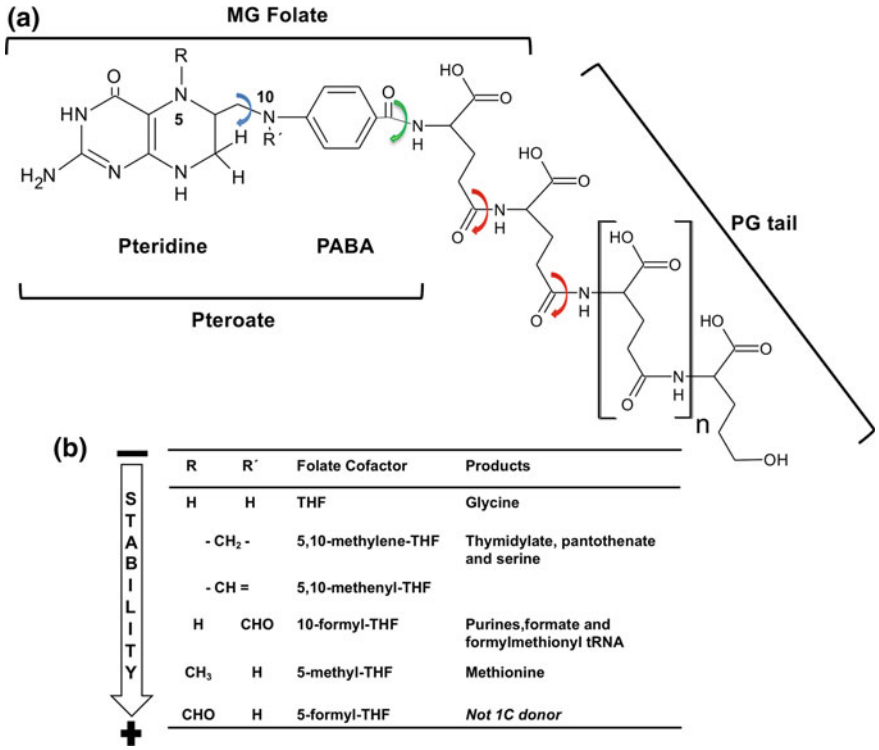


Fig. 6.1 **a** Chemical structure and breakdown sites of mono- (MG) and polyglutamylated (PG) folate molecule. Arrows indicate breakdown sites: oxidation of C9–N10 bond (blue), bond (green) cleaved by PABA hydrolase, and cleavage sites of two γ-glutamyl hydrolases (red). **b** Stability and functions of folate derivatives. *p*-aminobenzoate (PABA). Figure adapted from Ramos-Parra et al. (2013) and García-Salinas et al. (2016)

6.1.1 *Impacts of Folate Deficiency on Human Health and Folic Acid Fortification*

Folate malnutrition during early pregnancy increases the risk of congenital anomalies, mainly neural tube defects (NTDs). NTD can cause death in children five years old or younger or lifelong disabilities (Bailey et al. 2015). In addition to vitamin B12 deficiency, severe folate deficiency is also one of the causes of megaloblastic anemia (Rush 2000). Folate deficiency has also been associated with cardiovascular disease and some types of cancer (McNulty and Scott 2008). Also, recent studies correlate low folate status with cognitive dysfunction in elderly people (Araújo et al. 2015).

The established recommended dietary allowance (RDA) of folates is 400 mg/day for adults; during pregnancy, women need to consume 600 mg/day (FNB 1998). However, folate contents in foods vary depending on species, tissues, and developmental stages (Fig. 6.2). Leafy vegetables are good sources of folates, containing up to 200 μg/100 g, while legume seeds can accumulate very high folate levels (up to 600 μg/100 g of raw seeds). Staple crops, on the other hand, accumulate very low levels of the vitamin (<40 μg/100 g) (USDA 2018). Not all populations consume the RDA requirements in their regular diets; thus, more than 50 countries fortify flours with folic acid (Zimmerman 2011). Folic acid is the fully oxidized synthetic form of folate: It cannot act as a 1C donor, but it can be used as a supplement because it is reduced by dihydrofolate reductase (DHFR) in a two-step reduction that yields

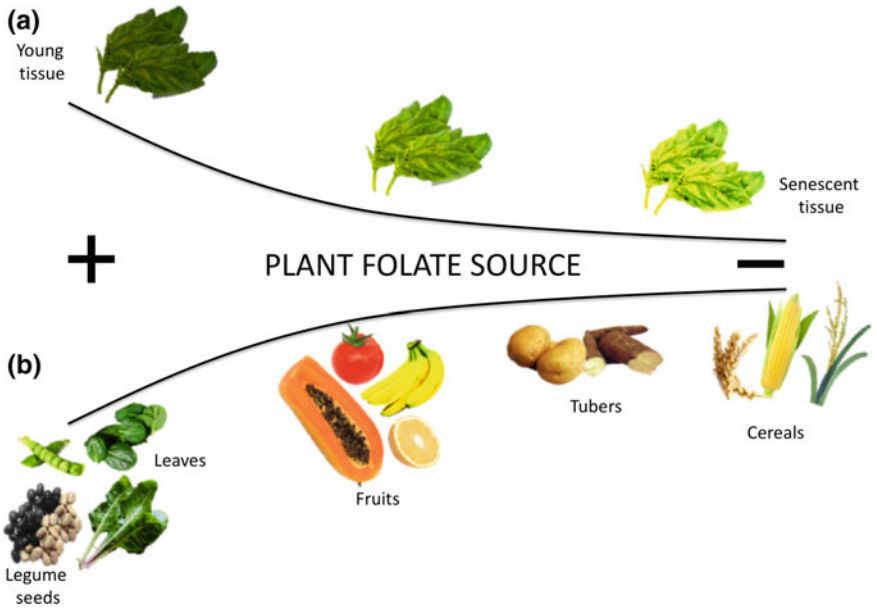


Fig. 6.2 Relative folate levels in plant foods. **a** Folate variations during development. **b** Folate contents in different plant tissues

THF (Fig. 6.3). Folic acid fortification has significantly diminished NTDs in the countries in which it has been implemented (Quinlivan and Gregory 2007); it is considered to be one of the best nutritional interventions worldwide. However, more than 300,000 children are born with NTDs every year globally; of these, 190,000 cases occur in low- and medium-income countries (Lo et al. 2014). It was estimated that in 2015, there were 117,900 NTD-associated deaths in children under 5 years old (Blencowe et al. 2018). These numbers are evidence that, in addition to folic acid interventions, alternative efforts for supplying folates are needed, primarily in the countries and regions in which folic acid fortification and supplementation are difficult to implement.

Alternatively, when folic acid is consumed in excess, it cannot be completely reduced by DHFR, and non-metabolized folic acid has been found in blood circulation (Plumptre et al. 2015). There are concerns about the possible adverse effects of chronic excessive intake of the synthetic folate in human metabolism (Lucock and Yates 2009). In addition, the consumption of high doses of folic acid is known to mask a vitamin B12 deficiency, which can cause neurological complications (Johnson 2007). In fact, some developed countries, mostly those in Europe, do not mandate folic acid fortification due to these concerns.

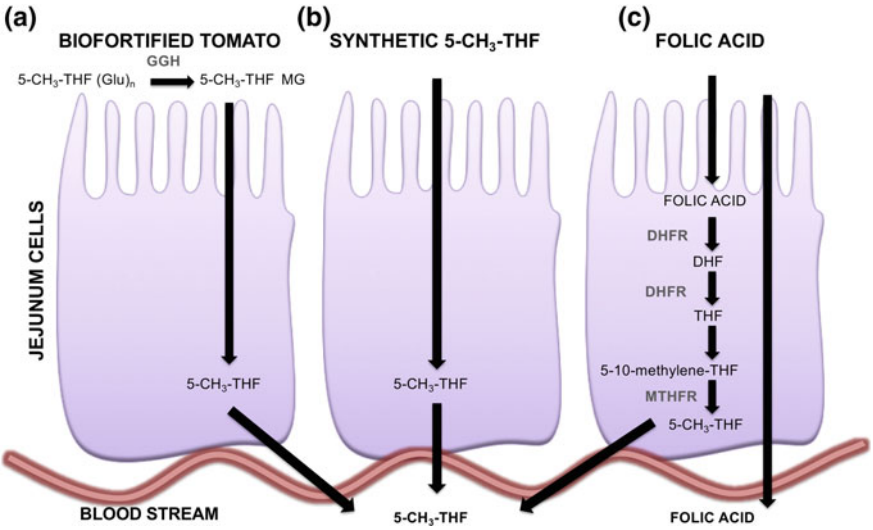


Fig. 6.3 Processing and absorption of synthetic and natural folates. **a** Polyglutamylated natural folates (mainly 5-CH_3-THF) are hydrolyzed by γ-glutamyl hydrolase (GGH, conjugase) in the intestinal lumen or at the brush border. Monoglutamylated (MG) folates are transported into the intestinal cell, appearing in the circulation as 5-CH_3-THF MG. **b** Synthetic 5-CH_3-THF MG is transported directly into the bloodstream. **c** Folic acid (FA) is transported into the intestinal cell, where it is reduced and methylated, appearing in the circulation as 5-CH_3-THF MG. Unmetabolized FA was observed in mesenteric circulation. DHFR, dihydrofolate reductase; MTHFR, 5,10-CH_2-THF reductase. Figure obtained from Castorena-Torres et al. (2014)

Biofortified crops accumulate reduced folates, mainly 5-CH_3-THF forms, and specific concerns regarding folic acid do not apply to this reduced folate form (Obeid et al. 2013). Moreover, biofortified crops can potentially reach broader populations where traditional fortification cannot be implemented. Biofortification of plant foods with folates has therefore become an attractive alternative for improving folate nutrition. In this chapter, all folate biofortification efforts in crops are covered, along with the biochemistry and chemistry of folates in plants and 1C metabolism.

6.2 Folate Metabolism in Plants

Folate biosynthesis and 1C metabolism are highly compartmentalized in plants (Figs. 6.4 and 6.5). Nearly all folate biosynthetic genes have been cloned in Arabidopsis, and a few of them have been characterized in plant crops. Each of the moieties comprising the folate molecule is synthesized in different compartments, as described in the following section.

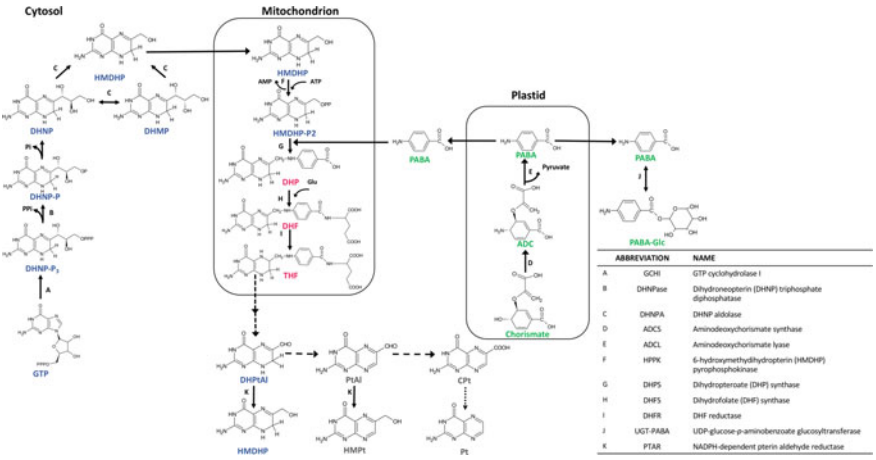


Fig. 6.4 Tetrahydrofolate biosynthesis and pteridine degradation products in plant cells. ADC, 4-amino-4-deoxychorismate; CPt, 6-carboxypterin; DHF, dihydrofolate; DHNP, dihydroneopterin; DNHP-P_3, DHNP, triphosphate; DHP, dihydropteroate; DHPtAl, dihydropterin-6-aldehyde; GTP, guanosine-5′-triphosphate; HMDHP, 6-hydroxymethyldihydropterin; HMPt, 6-hydroxymethylpterin; PABA, *p*-aminobenzoate; PABA-Glc, PABA β-D-glucopyranosyl ester; Pt, pterin; PtAl, pterin 6-aldehyde; THF, tetrahydrofolate. Dashed arrows indicate photochemical oxidation steps; dotted arrows indicate possible oxidation. Biosynthetic pteridines (blue); oxidized non-biosynthetic pteridines (gray); PABA branch (green); pteridine and PABA condensation products (magenta). Figure adapted from Ramírez-Rivera et al. (2016)

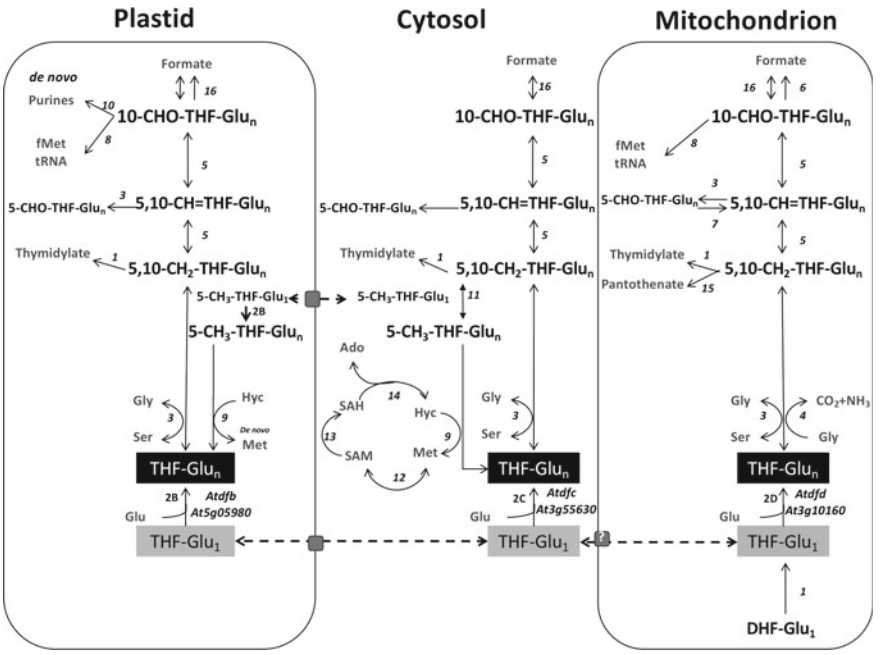


Fig. 6.5 Interconversion of folate derivatives and one-carbon (1C) metabolism reactions within different compartments of the plant cell. Arrows and numbers represent enzymatic reactions. Dashed arrows and gray squares represent folate transport and transporters respectively. 1. Dihydrofolate reductase/thymidylate synthase; 2. Folylpolyglutamylsynthetase (FPGS B, C and D isoforms); 3. Serine hydroxymethyltransferase; 4. Glycine decarboxylase; 5. 5,10-CH_2-THF dehydrogenase/5,10-CH=THF cyclohydrolase; 6. 10-CHO-THF deformylase; 7. 5-CHO-THF cycloligase; 8. Methionyl-tRNA formyltransferase; 9. Methionine synthase; 10. Glycinamide ribonucleotide (GAR) transformylase and aminoimidazole carboxamide ribonucleotide (AICAR) transformylase; 11. 5,10-CH_2-THF reductase; 12. S-adenosyl methionine synthetase; 13. Methionine S-methyltransferase; 14. S-adenosyl homocysteine hydrolase; 15. Ketopantoate hydroxymethyltransferase; 16. 10-CHO-THF synthetase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine. Figure adapted from Srivastava et al. (2011)

6.2.1 Biosynthesis Pathway

6.2.1.1 Cytosolic Branch

The pteridine moiety of a folate molecule is likely produced in cytosol from GTP; none of the characterized enzymes involved in pteridine production contain an evident localization signal. The first committed step for pteridine synthesis is catalyzed by GTP cyclohydrolase I (GCHI), which, in a complex cyclization reaction, converts GTP to the first pteridine of the pathway, dihydroneopterin triphosphate (DHNP-P_3). GCHI in plants is different from that of prokaryotes and vertebrates; in the latter, it starts the tetrahydrobiopterin synthesis instead of folates. The plant enzyme contains two GCHI-like domains, both indispensable for enzyme activity

(Basset et al. 2002). In tomatoes, GCHI expression declines as the fruit ripens, and the protein has not been detected in ripe fruit. This coincides with a slight decline in 5-CH_3-THF levels. Recently, two GCHI isoforms from soy were localized in the cytosol of Arabidopsis protoplasts by fusing the proteins with GFP, confirming the presumed cytosolic localization of the enzyme (Liang et al. 2019).

DHNP-P_3 is dephosphorylated in two steps; it is not clear yet if there are enzymes committed solely to processing DHNP-P_3, or if general pyrophosphorylases and phosphatases could use the phosphorylated pteridine as a substrate. First, the pyrophosphate is removed from DHNP-P_3; Arabidopsis has a Nudix hydrolase that is able to hydrolyze DHNP-P_3 in vitro in addition to other substrates. Thus, its participation in folate biosynthesis in vivo has yet to be confirmed (Klaus et al. 2005b). DHNP-P could then be dephosphorylated to DHNP by a phosphatase(s) that has yet to be discovered.

DHNP is the substrate of dihydroneopterin aldolase (DHNPA), the Arabidopsis genome contains three copies of these genes; two of them have been characterized in Arabidopsis, along with one gene in tomatoes (Goyer et al. 2004). The plant enzyme has two activities: the aldolase and also an epimerase that converts DHNP to dihydromonapterin (DHMP). DHNPA is able to take both isomers as substrates, breaking them down to hydroxymethyldihydropterin (HMDHP) and glycosides. HMDHP is then transported into the mitochondria by a characterized carrier that has yet to be identified.

6.2.1.2 Plastidial Branch

Plastids are the site of PABA biosynthesis, starting with the 4-amino-4-deoxychorismate (ADC) production by ADC synthase (ADCS), which commits chorismate for folate synthesis by catalyzing two reactions: NH_3 release from glutamine and the hydroxyl group replacement with the amino group yielding ADC. This enzyme possesses a plastidial transit peptide, and it can be inhibited by dihydrofolate and methotrexate; fully reduced folates and PABA do not affect the activity of the enzyme (Basset et al. 2004; Sahr et al. 2006). ADC is a labile molecule that might be spontaneously converted to PABA to some extent and is also the substrate of ADC lyase (ADCL), which releases pyruvate from ADC, yielding PABA. As with GCHI, ADCS, and ADCL, mRNA levels drop as tomato fruits ripen, while PABA levels increase at the onset of ripening and slightly decrease in ripe red fruit. PABA is a weak acid that most likely can cross membranes by diffusion; therefore, it is possible that this moiety is able to cross the plastidial and mitochondrial membranes to participate in folate biosynthesis. In fact, the PABA pool in plants is composed of free PABA and esterified PABA with glucose (PABA-Glc) (Eudes et al. 2008; Quinlivan et al. 2003). PABA esterification is reversible in the cytosol, mediated by an UDP-glucosyltransferase (UGT75B1 in Arabidopsis). Remarkably, PABA-Glc is mainly found within the vacuole, not into the mitochondria (Eudes et al. 2008, Fig. 6.6). Thus, this PABA ester can be a form of regulating the diffusible PABA; PABA-Glc is thus proposed to be a storage and sequestrable form.

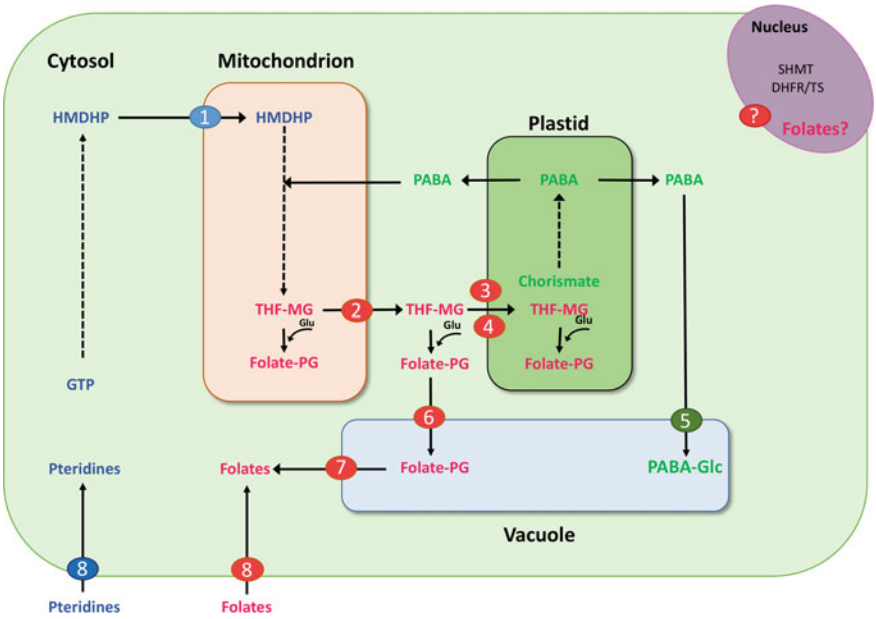


Fig. 6.6 Transport of folates and its precursors among plant cell compartments. The known or presumed transporters are indicated with circles; only 3, 4, and 7 have been cloned. DHR/TS, dihydrofolate reductase/thymidylate synthase; GTP, guanosine-5′-triphosphate; HMDHP, 6-hydroxymethyldihydropterin; MG, monoglutamyl; PABA, *p*-aminobenzoate; PABA-Glc, PABA β-D-glucopyranosyl ester; PG, polyglutamyl; SHMT, serine hydroxymethyl transferase; THF, tetrahydrofolate. Figure adapted from Hanson and Gregory (2011)

6.2.1.3 Folate Production in Mitochondria

Once HMDHP is located in the mitochondria, it is phosphorylated by HMDHP pyrophosphokinase (HPPK), which uses ATP to form HMDHP pyrophosphate (HMDHP-P_2). Then, this moiety is condensated with PABA to form dihydropteroate (DHP) by DHP synthase (DHPS). Both activities are present in plants in a bifunctional protein, HPPK-DHPS, which comprises the two domains. This enzyme is strongly inhibited in vitro by DHP, DHF, and monoglutamylated THF (Mouillon et al. 2002).

Arabidopsis is the only plant reported to have a cytosolic form of HPPK-DHPS, but it does not seem to be involved in folate synthesis; instead, it is likely related to an abiotic stress resistance mechanism (Navarrete et al. 2012). DHP produced in cytosol would not serve for folate production in this compartment, as it would need to enter the mitochondria for this purpose. In mitochondria, DHP is glutamylated by dihydrofolate synthase (DHFS) to form the first folate of the pathway, dihydrofolate (DHF) (Ravanel et al. 2001). Arabidopsis has one single DHFS gene, the function of which has been characterized genetically; DHFS partial disruption causes defective

embryo development that could be rescued by exogenously supplying an excess of folate (Ishikawa et al. 2003).

DHF does not participate in 1C reactions; in fact, it inhibits some. DHF has to be further reduced to THF by DHFR into the mitochondria in order to serve as a cofactor. DHFR in plants is another bifunctional protein with thymidylate synthase (TS) activity (Lazar et al. 1993). The coupling of these two activities can be advantageous, as TS uses 5,10-CH_2-THF as both methyl donor and a reducing agent to form thymidylate, yielding DHF. This oxidized folate can be channeled and reduced immediately by the DHFR domain, which uses NADPH as a reductant. Therefore, mitochondria are the site in which THF, the first folate derivative, is synthesized de novo.

6.2.2 Folate Polyglutamylation: Regulation of Compartmentalization and 1C Reactions

THF and other folate derivatives produced in mitochondria need to be transported to other cell compartments (Fig. 6.6). However, to date; very few transporters have been cloned and characterized in plants, none of them targeted to the mitochondrial membrane. Monoglutamylated (MG) folates are the preferred folate forms by transporters (Hanson and Gregory 2011). Folate retention within the cell and compartments is thus partially regulated by the addition of glutamyl moieties to the cofactor. Folylpolyglutamate synthase (FPGS) is the enzyme that adds glutamate units to the molecule (Ravanel et al. 2001).

There are three FPGS enzymes within plant cells, localized into mitochondria, cytosol, and plastids (Fig. 6.5). Polyglutamylate (PG) folate forms have been found within the three compartments and also within the vacuole. However, a plant's vacuole contains an enzyme that cleaves this PG tail: γ-glutamyl hydrolase (GGH) (Orsomando et al. 2005). There are three isoforms with different activities. GGH1 activity yields mainly diglutamylated folates, while GGH2 deglutamylates folates to their monoglutamyl forms. GGH3 activity has not been assessed in Arabidopsis (Orsomando et al. 2005), and no activity has been detected for the tomato homolog (Akhtar et al. 2008).

The fact that vacuoles contain PG 5-CH_3-THF is surprising (Orsomando et al. 2005; Akhtar et al. 2008); as no FPGS is known to be located in the vacuole, these folate forms need to be transported into the vacuole and also protected from GGH activity. To date, an ATP-binding cassette transporter that is able to translocate methotrexate (a folate analogue) is the only cloned possible folate transporter targeted to the tonoplast; it probably transports MG folates into the vacuole (Raichaudhuri et al. 2009). However, more transporters would need to be present in the tonoplast to sustain the PG folate derivatives observed within the vacuole (Fig. 6.6). Regarding the other folate transporters studied in plants, in the chloroplast envelope of Arabidopsis, only two proteins have been characterized to date: At5g66380 and At2g32040

(Bedhomme et al. 2005; Klaus et al. 2005a). Both have the capacity to transport MG folates in vitro and probably have redundant functions, as individual loss of function mutants have not displayed a growth phenotype. Nevertheless, the At2g32040 mutant alters folate distribution and accumulation within chloroplasts (Klaus et al. 2005a).

The PG tail then plays a key role in regulating folate pools and distribution within the cell. Evidence for this has been provided by the loss of function in individual FPGS in Arabidopsis. Individual knockouts (KOs) have caused alterations in folate derivatives distribution in the affected compartment, and, in the case of plastid and mitochondrial isoforms, loss of function of each specific FPGS caused a folate leakage within these compartments. Moreover, individual and double FPGS loss of function mutants have shown a myriad of developmental and metabolic consequences (Akhtar et al. 2010; Mehrshahi et al. 2010; Srivastava et al. 2011; Waller et al. 2009). This can be partially explained by less folate molecules available to accept and donate 1C units within compartments, but also a short PG tail can signify less folate affinity when used as a cofactor by the 1C metabolism enzymes.

Some of the folate-utilizing enzymes have more affinity to PG folates than to MG derivatives. The best example is methionine synthase (MS), which practically does not use mono- and diglutamylated 5-CH_3-THF as cofactors, and it has a better affinity with the triglutamylated form (Ravanel et al. 2004). De novo methionine biosynthesis occurs in plastids, and loss of function of plastidial FPGS has remarkably led to multiple developmental and metabolic phenotypes recently described in Arabidopsis: DNA hypomethylation, perturbations in nitrogen metabolism, root development, lignin composition, and starch homeostasis in plastids (Hayashi et al. 2017; Mehrshahi et al. 2010; Meng et al. 2014; Reyes-Hernández et al. 2014; Srivastava et al. 2011; Zhou et al. 2013). More evidence regarding the importance of the folate PG degree comes with the modulation of GGH activity; its overexpression led to less folate accumulation in tomato fruits (Akhtar et al. 2010), while its silencing increased folates by 34% (Akhtar et al. 2008). Thus, polyglutamylation of folates is a form of regulation of folate distribution and accumulation among cell compartments and also of 1C metabolism.

6.2.3 Folate Interconversion and 1C Reactions: Compartmentation Is Key

Folates derivatives are not distributed equally among cell compartments (Chen et al. 1997; Orsomando et al. 2005); this is due in part to the reactions that utilize folates as cofactors causing their methylation, reduction, and oxidation occur unequally among compartments. THF produced in mitochondria and generated by 1C reactions in other compartments obtains the 1C unit from serine by the action of serine hydroxymethyl transferase (SHMT), which produces 5,10-CH_2-THF in a reversible reaction. During

mitochondrial photorespiration, the 1C unit can also come from glycine by the glycine decarboxylase complex in mitochondria (Fig. 6.5).

The Arabidopsis genome contains seven SHMT isoforms that have been localized in mitochondria, plastids, cytosol, and nuclei (Hanson and Roje 2001; Zhang et al. 2010; Wei et al. 2013). The hydroxymethyl group from serine is transferred to THF, yielding glycine; conversely, 5,10-CH_2-THF can be taken by SHMT to form serine from glycine. SHMT reaction equilibrium favors glycine formation; serine then becomes a major 1C donor in organisms.

Mitochondrial, plastidial, and cytosolic SHMT isoforms have been biochemically characterized, showing different affinities for MG and PG folates, PG folates being better substrates (Rebeille et al. 1994; Wei et al. 2013; Zhang et al. 2010). 5-CHO-THF and 5-CH_3-THF inhibit SHMT, and PG forms show more affinity than MG for the plastidial SHMT isoform (Zhang et al. 2010). As SHMT regulates a crucial 1C entry point and is essential for photorespiration in mitochondria, its activity has to be modulated by multiple factors; in addition, the distinct characteristics found among the multiple isoforms hint at a complex metabolic regulation at many levels. In fact, one of the mitochondrial isoforms, SHMT1, is the target of ubiquitin conjugation, which tags proteins for proteasome-dependent degradation (Zhou et al. 2012). Less SHMT1 activity leads to ROS overaccumulation and salt stress sensitivity (Moreno et al. 2005). Changes in SHMT1 accumulation and activity due to ubiquitin removal are correlated with Na^+/H^+ antiport activity, providing salt tolerance in Arabidopsis, probably due to less ROS production. This exemplifies how products and byproducts of 1C metabolism can broadly impact plant physiology.

5,10-CH_2-THF is oxidized to 5,10-CH=THF and then to 10-CHO-THF in these three compartments by the bifunctional and reversible 5,10-CH_2-THF dehydrogenase/5,10-CH=THF cyclohydrolase (5,10MTHFD/C) isoforms. This bifunctional, NADP-dependent protein has been characterized in pea cotyledons and leaves crude extracts; high activities were localized in the cytosol and minor ones in mitochondria (Besson et al. 1993). Forward genetic studies have shown that 5,10MTHFD/C activities are crucial in plants. Partial loss of function of the cytosolic isoform in Arabidopsis presented as an effect of DNA hypomethylation and transposon derepression, while complete loss of function proved lethal (Groth et al. 2016).

10-CHO-THF can be converted to formate and THF by10-CHO-THF deformylase (10-FDF). The Arabidopsis genome contains two genes that code for two isoforms both localized in the mitochondria (Collakova et al. 2008). The double knockout (KO) of these genes results in developmental abnormalities and affects sugar, lipid, and amino acid metabolisms. These phenotypes vary depending on photorespiratory conditions: When the plants are in non-photorespiratory environments, phenotypes are rescued, while in high CO_2 levels, double KO is lethal. Mitochondrial folates have a crucial role during photorespiration, where glycine provides 1C unit to THF to form 5,10-CH_2-THF; this transfer is mediated by the glycine decarboxylase complex (GDC). During photorespiration, GDC activity shifts the SHMT equilibrium favoring serine synthesis (Rebeille et al. 1994), forming THF and serine. This flux is massive, mostly in C_3 plants, and needs to be taken into account when 1C mutants are characterized.

Additionally, during photorespiration, SHMT activity produces the only folate derivative that is not a 1C cofactor, 5-CHO-THF, which is an intriguing molecule. It cannot accept or donate 1C; in fact, it inhibits some of the 1C metabolism enzymes (e.g., the same complex that produces it, glycine decarboxylase/SHMT), but it is normally accumulated within the plant cell, mainly in the mitochondria (Chen et al. 1997; Orsomando et al. 2005). Moreover, it has been widely used in large amounts (0.5–10 mM) to chemically rescue the majority of the Arabidopsis 1C metabolism-related mutants, as it is absorbed by the plant cell and roots, and is, surprisingly, easily converted to the active cofactors in plant cells and tissues (Srivastava et al. 2011). Mitochondrial 5-CHO-THF cycloligase (5-FCL) is the enzyme that converts it to 5,10-CH=THF; this folate form can be further reduced to 5,10-CH_2-THF by 5,10MTHFD/C or oxidized by the same enzyme in the opposite direction to 10-CHO-THF (Goyer et al. 2005; Roje et al. 2002). 10-CHO-THF can also be produced by 10-CHO-THF synthetase, which uses formate and ATP to add the formyl group to the THF and is also reversible. Formate, along with serine and glycine (during photorespiration), then becomes another 1C source by the reaction toward 10-CHO-THF biosynthesis. In mammals and yeast, this activity is present in a trifunctional protein known as C1-THF synthase, which also contains the 5,10MTHFD/C activities. 1C metabolism in plants differs from that of mammals, as a separate protein contains the 10-CHO-THF synthetase activity. In early studies, this enzyme was cloned, isolated, and characterized from spinach and pea leaf extracts from subcellular compartments (Chen et al. 1997; Nour and Rabinowitz 1991, 1992). The majority of the activity was found in the cytosolic fraction of pea leaves; little was found in the mitochondria, and none was detected in plastids under the conditions tested. However, plants might have isoforms in the three compartments.

Cytosol is the only place in which 5,10-CH_2-THF can be reduced, as 5,10-methylene-THF reductase (MTHFR) two isoforms in Arabidopsis seem to lack a targeting signal to other cell compartment (Roje et al. 1999). MTHFR produces the 5-CH_3-THF cofactor, the only known fate of which is to be used to methylate homocysteine for methionine synthesis by MS. Besides polypeptide synthesis, the majority of the methionine pool is used as the precursor of S-adenosylmethionine (SAM), which is the universal methyl donor in organisms. 5-CH_3-THF is also the main folate found in plant tissues and the blood circulating folate form for mammals (Bedhomme et al. 2005). This evidences the relevance and magnitude of this 1C reaction for organisms, which is considered to represent the highest flux of 1C metabolism (Hanson and Roje 2001). Recently, MTHFR maize mutants were isolated by forward genetics; they displayed reduced transcript levels, which impacted lignin production, mainly G lignin biosynthesis, accumulating less lignin with an altered composition (Tang et al. 2014; Wu et al. 2018). Additionally, changes in the expression of MTHFR triggered opposite alterations in the expression profile of a nicotine *N*-demethylase gene, CYP82E4, with a concomitant change in alkaloid production and profiles in tobacco leaves (*Nicotiana tabacum*) (Hung et al. 2013).

All folate cofactors are used in anabolic reactions. In plastids, 10-CHO-THF is used for de novo purine biosynthesis in two transformylation reactions mediated by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and

5-formaminoimidazole-4-carboxamide ribonucleotide (FAICAR) (Zrenner et al. 2006). De novo methionine biosynthesis also occurs in this compartment, mediated by MS1 (Ravanel et al. 2004). The mitochondria are the site of the first committed step for pantothenate (vitamin B5) production; ketopantoate hydroxymethyltransferase (KPHMT) utilizes 5,10-CH_2-THF to produce the ketopantoate. The Arabidopsis genome contains two genes coding for KPHMT, both of which are mitochondria localized (Ottenhof et al. 2004). Bacteria, mitochondria, and plastids initiate protein synthesis using formyl methionine tRNAs produced by methionyl-tRNA formyl transferase that uses 10-CHO-THF as the formyl donor (Kozak 1983). This enzyme has yet to be characterized in plants.

Thymidylate is produced using 5,10-CH_2-THF to reduce deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by TS, which in plants is fused to DHFR (see above) (Lazar et al. 1993). Recently, three Arabidopsis DHFR-TS gene products (DHFR-TS1-3) were localized into the cytosol (DHFR-TS1), mitochondria (DHFR-TS2), and both compartments (DHFR-TS3). All three isoforms were also detected in nuclei in specific cells; their localization was dependent on the cell's differentiation state (Gorelova et al. 2017). Interestingly, DHFR-TS3 did not present DHFR and TS activities. On the contrary, its expression caused inhibition of both DHFR-TS1 and 2; this effect prompted elevation of reactive oxygen species (ROS) that led to recognize the activity of this enzyme, and thus folate-mediated 1C metabolism, as factors for NADPH production sustainability and concomitant redox homeostasis in plant cells. This study also confirmed the localization of some of the folate-utilizing enzymes within the nuclei. Previously, SHMT and MTHFD activities were detected in plant nuclei extracts (Neuburger et al. 1996); thus, a folate pool must exist in this compartment in plants (Fig. 6.6). Folate presence has been previously reported in mammalian cell nuclei; folate cofactors are utilized by SHMT, DHFR, and TS to provide thymidylate for DNA synthesis. These enzymes are imported into the nucleus during the S-phase of the cell cycle (Anderson and Stover 2009; Palmer et al. 2017). Folates and 1C metabolism have yet to be studied in plant nuclei.

6.3 Folate Degradation, Turnover, and Stability in Plant Food Matrices

Reduced folates are chemically very labile molecules that are protected in vivo by the 1C transfer enzymes within the cell (Rebeille et al. 1994). Folates are prone to photooxidation, mostly the breakage of the C^9–N^{10} bond. This breakage occurs spontaneously, yielding a pterin and PABA-MG or PG; it is not known if plants possess an enzyme to mediate folate degradation. The presence of the 1C unit and its level of oxidation strongly affect the stability of the molecule. Folic acid, the fully oxidized synthetic vitamin, is very stable; by comparison, THF, the fully reduced folate, is the most labile form (Fig. 6.1). As many of the enzymes that use folate

cofactors have more affinity to the PG forms, these forms have been proposed to be more stable within the cell. In fact, the folate increases achieved when engineering a longer PG tail support this previous assumption (Akhtar et al. 2008).

The pterin product of folate breakdown can be recycled for folate synthesis only if it is properly reduced; dihydropterin-6-aldehyde is the substrate of pterin aldehyde reductase (PTAR), which can reduce it to HMDHP that can then be incorporated into the mitochondrial steps of the folate biosynthesis (Noiriel et al. 2007a; Orsomando et al. 2006). On the other hand, if the pterin ring is further oxidized, these pterins cannot be recycled back into the biosynthetic pathway (Fig. 6.4, Noiriel et al. 2007b). Assuming that the PABA forms can enter and exit the vacuole, PABA-PG can be a substrate for plant GGHs (Orsomando et al. 2005), which yield PABA-MG. PABA-MG is a substrate of PABA glutamate hydrolase (PGH), which releases the PABA moiety back into the pathway; this activity has been confirmed in plants and likely has various isoforms, but the gene has not yet been cloned (Bozzo et al. 2008).

There are several works that have studied the stability of folates within plant food matrices during food processing. Both house and industrial processing usually cause folate loss. The extent of the loss is highly dependent on the matrix and conditions. The main mechanism responsible for folate loss is leaching to a liquid surrounding the food. Temperature can also negatively affect folates, and this is highly dependent on the pH of the food matrix or media, as folates are very susceptible to oxidation at low pH levels. Thus, extensive folate stability studies have been conducted on a case-by-case basis. A very comprehensive review of folate stability in plant foods was done by Delchier et al. (2016). Here, a short selection of representative works is summarized in Table 6.1 to give a perspective of the most common effects of processing on plant foods. It is interesting to note that in many of the works using high pressures, which causes cell damage and membrane breakage, an extensive deglutamylation of folates was observed, perhaps due to the release of GGHs from the vacuole.

Unlike with processing, there are few studies evaluating folate dynamics during postharvest conditions. Folate contents were assessed in different varieties of strawberry fruits (*Fragaria* × *ananassa*) during storage. Variations were cultivar-dependent, and the temperature of storage had a considerable effect on folate stability, with low temperatures increasing folate retention (up to 99% at 4 °C), while storage at 20 °C caused a steep decline in folate contents (38% loss in 3 days) (Strålsjö et al. 2003). Contrary to fruit, which can only be stored for a certain number of days, storage of tubers and seeds lasts for months. In the case of potato tubers, 7 months of cold storage had a positive effect, causing increases up to 1.78-fold. These increases were variety-dependent, and it was suggested that folates may be increased as preparation for sprouting, as has been observed in the germination of wheat seeds (Goyer and Navarrete 2007; Koehler et al. 2007).

However, rice seeds stored at room temperature for one year lost an average of 23% of folate contents. This was also cultivar-dependent: The variety that lost the highest amount of folates retained only 43.5% of the original folate contents (Dong et al. 2011). The relevance of postharvest conditions was even more evident in biofortified rice. Even after a successful biofortification process, enhanced folates in rice suffered

Table 6.1 Effect of processing on folate stability of plant food matrixes

Treatment	Plant food matrix	Process effect on folates	Comment	References
Legumes				
Industrial canning soaking, 20 °C/14 h blanching 98 °C/2 min autoclaving 128 °C/5 min	Faba beans	51% ↑ soaking 15% ↓ blanching 23% ↓autoclaving	Increased folates by soaking were lost during blanching and autoclaving. 5-CH_3-THF was the folate derivative that significantly changed during processes	Hefni and Witthöft (2014)
	Chickpeas	51%↑ soaking 31% ↓blanching 44% ↓autoclaving		
Soaking for 16 h, following boiling 2 h or pressure cooking for 20 min	Chickpeas	47% ↓ boiling 38%↓ pressure-cooked	Boiling caused more folate leaching than pressure cooking in both legumes. Folates were detected in cooking medium	Dang et al. (2000)
	Peas	55% ↓ boiling 50% ↓ pressure-cooked		
Canning 125–130 °C, 6–15 min	Green beans	30% ↓	Losses due to leaching	Delchier et al. (2013)
Blanching 100 °C water/10 min	Green beans	21%↓	Losses due to leaching	Melse-Boonstra et al. (2002)
200 MPa, 5 min	Green beans	47% ↓ total folate	Folate leakage during HHP treatment	
300, 450 and 600 MPa, 30 °C, 5 and 10 min (HHP)	Green beans	18% ↓ at 600 MPa, 10 min 20% ↑ at 450 MPa/5 min	Pressure and time increased folate deglutamylation in all cases with increases in MG folates from 10 to 48%	Luo et al. (2017)
	Yardlong beans	35% ↓ 600 MPa, 10 min 11% ↑ 300 MPa, 5 min		

(continued)

Table 6.1 (continued)

Treatment	Plant food matrix	Process effect on folates	Comment	References
	Winged beans	6% ↓ 600 MPa, 10 min		
Cereals				
Boiling water 30 min	Milled rice (8 varieties)	38–69% ↓	Significant differences in folate stability among rice varieties	Dong et al. (2011)
Boiling/15 min steaming/30 min	Amaranth Quinoa Buckwheat	58 and 22% ↓ boiling and steaming 15↑and ≈ boiling and steaming ≈	When folates were expressed in dietary reference values, no significant change was observed among the treatments	Motta et al. (2017)
Vegetables				
Freezing	Spinach	25% ↓	Losses due to leaching	Delchier et al. (2013)
95 °C/10 min (HTST)	Broccoli	≈	PG folates percentage increased after treatment	Munyaka et al. (2009)
60 °C/40 min (LTLT)	Broccoli	≈	MG folates percentage increased after treatment	
0.1–600 MPa, 25–45 °C, 30 min (HHP)	Broccoli	≈5-CH_3-THF (25–45 °C, ≤200 MPa) 48–78% ↓ 5-CH_3-THF when >300 MPa were applied	Increasing both pressure and temperature decreased 5-CH_3-THF contents and PG levels	Verlinde et al. (2008)
Blanching 100 °C water/10 min	Leeks	28% ↓	Losses due to leaching	Melse-Boonstra et al. (2002)
200 MPa, 5 min (HHP)		81% ↓ total folate 41% ↑ MG	Leakage of folate during HHP treatment	

(continued)

Table 6.1 (continued)

Treatment	Plant food matrix	Process effect on folates	Comment	References
Blanching 100 °C water/10 min	Cauliflower	10% ↓	Losses due to leaching	
200 MPa, 5 min (HHP)		43% ↓ total folate 3% ↑ MG	Leakage of folate during HHP treatment	
300, 450, 600 MPa, 30 °C, 5 min (HHP)	Cauliflower	≈5-CH_3-THF ~40% and 22% MG ↑ at 300 and 600 MPa	GGH was inactivated by 10-min steaming prior treatment	Wang et al. (2011)
	Carrot greens	32% ↓ 5-CH_3-THF at 450 MPa 50% ↓ 5-CH_3-THF at 600 MPa		
Fruits				
Pasteurization at 98, 108 and 128 °C for 40 s	Tomato puree	35% ↑5-CH_3-THF at 98 °C ≈5-CH_3-THF at 108 °C 15% ↓ 5-CH_3-THF at 128 °C	Possible increase in folate extractability folate at 98 °C Folates were more labile at >108 °C	Iniesta et al. (2009)
600 MPa, 25/80 °C, 5 min	Orange juice	≈5-CH_3-THF and 5-CHO-THF 20% ↓ THF at 25 °C 15% ↓ 5-CH_3-THF 35% ↓ 5-CHO-THF 15% ↓ THF at 80 °C	Folates from freshly squeezed orange juice were significantly more stable than in a model juice with the same ascorbic acid contents	Butz et al. (2004)
50–200 MPa, 25 °C, 10 and 30 min/500 MPa, 60 °C, 5–100 min	Orange juice	≈5-CH_3-THF	Endogenous ascorbate contributes to folate stability during processes	Indrawati et al. (2004)

(continued)

Table 6.1 (continued)

Treatment	Plant food matrix	Process effect on folates	Comment	References
Roots and tubers				
Boiling water, 60 min	Potato	≈total folate	Peeling potato skin before or after boiling did not affect folate contents	McKillop et al. (2002)
Sous vide process 100 °C, 35 min	Peeled potato	≈total folate 41% ↓	Folate retention was also affected by warm or cold storage and subsequent reheat	Stea et al. (2007)
Boiling water, 11 min				
Boiling water, 33 min	Unpeeled potato	28% ↓		
Oven-baked at 225 °C 80 min		37% ↓		
50–200 MPa, 25 °C, 10 and 30 min/500 MPa, 60 °C, 5–100 min	Carrot juice	8–33% ↓ 50–200 MPa, 25 °C 59–77% ↓ 500 MPa, 60 °C	Folate losses were prevented by ascorbic acid addition	Indrawati et al. (2004)
300 and 600 MPa, 30 °C, 5 min	Baby carrots	30% ↓ 5-CH_3-THF at 300 MPa	GGH was inactivated by 10-min steaming prior treatment	Wang et al. (2011)

MG, monoglutamylated; PG, polyglutamylated Glu, γ-glutamyl residues; THF, tetrahydrofolate; 5-CH_3-THF, 5-methyl-THF; 5-CHO-THF, 5-formyl-THF; HTST, high temperature short time; LTLT, low temperature long time; HHP, high hydrostatic pressure

large losses when biofortified seeds were stored for four months (Blancquaert et al. 2015). Little is known about folate homeostasis and dynamics during storage, which is unavoidable. In some plant organisms, biosynthesis could be occurring, while in others, changes in folate pools may be due to spontaneous or enzymatic degradation. These few studies also show that there is variation in the extent of folate stability among plant varieties; thus, plants could be bred for folate stability. These studies are critical for advancing biofortified varieties.

Studying folate homeostasis during postharvest processes is also relevant in the case of fruits because many are harvested at the mature green stage and then ripened during storage. There are a few reports that characterize folates during fruit ripening; however, the ripening occurred on the vine, which is a rare commercial practice. When papaya fruit was stored for up to 9 days at 21 °C and reached full ripening, total folates increased by 50% (Ramos-Parra et al. 2013). Conversely, postharvest ripening did not significantly affect folate levels for tomato, banana, and avocado fruits when compared to their mature green counterparts (García-Salinas et al. 2016). These four fruits are climacteric, which are characterized by an increase in respiration and ethylene biosynthesis during ripening (Lelievre et al. 1997). This characteristic is used commercially: Mature green fruit is treated with ethylene to trigger ripening before the exhibition on the shelf. These four fruits were also treated with exogenous ethylene, which significantly affected folate accumulation for certain fruit species. Papaya folate fruit contents decreased by 26% when fruit ripening was triggered by ethylene; conversely, total folates increased by 26 and 51% in tomato and banana fruits, respectively. In avocado fruits, no change was observed. However, exogenous ethylene application affected 5-CH_3-THF levels in the majority of the fruits tested, which is the folate form implicated in ethylene biosynthesis *in planta* through methionine synthesis (Ravanel et al. 2004). Methionine, through the formation of SAM, is an ethylene precursor. SAM is converted to aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and further oxidized to ethylene by ACC oxidase (Sauter et al. 2013). Thus, ethylene and folate metabolism have a cross-path and likely influence the homeostasis of each other. In fact, MS expression is upregulated at the transcriptional level in papaya, apple, and banana fruits after exogenous ethylene treatment (D'hont et al. 2012; Fabi et al. 2010; Zheng et al. 2013). Obtaining knowledge about this possible co-regulation has the potential to enhance folate contents in plant foods by adjusting postharvest conditions.

Other postharvest practices might be beneficial for increasing folates in plant foods; for example, spinach leaves stored at 4 °C under continuous light for up to 10 days accumulated more folates than those stored in complete darkness, where a decline was observed. The younger the leaves, more folates were produced (Lester et al. 2010). Similar effects were observed in pea leaves from etiolated seedlings when light-induced HPPK-DHPS gene expression (Jabrin et al. 2003). Folate accumulation has been correlated with photosynthesis through the need of methyl groups for chlorophyll biosynthesis and probably methylation of other substrates (Van Wilder et al. 2018). Interestingly, applying light to stored spinach leaves caused an average twofold increment in total folates (Lester et al. 2010). Spinach leaves are a good folate

source; thus, this easy, inexpensive practice would have the potential to contribute to folate nutrition and could work for other leafy plant foods.

6.4 Biofortification of Folates

The efforts to biofortify crops with folates began almost two decades ago; to date; all the biofortified lines are in the state of proof of concept and are the result of metabolic engineering. Efforts toward biofortification by breeding have been conducted by looking at natural variations in a few crops (Table 6.2). Overall, the results from these works show that the variations found to date are less than one order of magnitude; potato and rice germplasms have the largest range of folate levels for the edible tissue (Dong et al. 2011, 2014; Robinson et al. 2015).

Three studies linking folate to genome variations in tomato fruit, rice, and common bean seeds showed that the promising gene targets might not only be biosynthetic genes. A possible marker was identified from three concurrent quantitative trait loci (QTL) associated with folate contents in dry bean seeds (Khanal et al. 2013). In rice, three QTLs were identified, one of them explaining 25% of the phenotypic variation for folate contents; interestingly, none of them carried any of the known folate biosynthetic genes in rice (Dong et al. 2014). Moreover, the folate variation found in tomato fruits could not be associated with single-nucleotide polymorphisms (SNPs) from the biosynthetic genes (Upadhyaya et al. 2017). Characterization of those possible new markers will prompt better understanding of folate homeostasis in particular food crops. More studies were conducted to evaluate folate levels in relation to environmental changes, and folate contents varied significantly in strawberry fruits, rice, and wheat seeds according to the growing region and harvesting years (Dong et al. 2014; Kariluoto et al. 2010; Strålsjö et al. 2003). In fact, folate contents from wheat were considered to be affected more by the environment than by the genotype (Kariluoto et al. 2010). Thus, to generate accurate molecular markers for folate accumulation, as with other traits, the environment must be considered.

The lowering costs of genotyping will need to be met with simpler, faster, and more accurate folate analysis to be able to perform genome-wide association studies (GWAS) using several hundred samples (Luo 2015). GWAS will allow for the relatively quick identification of relevant SNPs linked to major QTLs that can become specific markers to accelerate the breeding process in staple crops for natural folate enhancement. To date, the data obtained demonstrates that this approach is promising for folate biofortification. To the authors' knowledge, there have not yet been works on any crop about breeding for folates. Another recent approach for improving folate contents has been elicitation either by plant hormones or folate precursors. Salicylic acid treatments doubled folate contents in coriander leaves (Puthusseri et al. 2013), while the addition of phenylalanine to hydroponically grown spinach elevated PABA around 35% and doubled folate production (Watanabe et al. 2017). This approach could work for vegetable production in controlled environments.

Table 6.2 Natural variation of folate accumulation in food crops

Crop	# Individuals screened	Folate contents µg 100 g^{-1} [a] (fold increase)	References
Rice seeds (*Oryza sativa* L.)	78 varieties	Brown rice 13.3–111.4 (**8.4**)	Dong et al. (2011)
		Milled rice 10.3–77.7 (**7.5**)	
Milled rice seeds (*Oryza sativa* L.)	264 recombinant inbred lines	10.0–62.5 (**6.25**) year 1	Dong et al. (2014)
		25.2–169.4 (**6.7**) year 2	
	182 backcross inbred lines	14.5–129.8 (**9**)	
Wheat grains (*Triticum aestivum*)	156 individuals 26 genotypes Multiple locations and seasons	32.3–88.9 dw (**2.8**)	Kariluoto et al. (2010)
Wheat grains (*Triticum aestivum*)	150 genotypes	Winter wheat 36.4–77.4 dw (**2.1**)	Piironen et al. (2008)
		Spring wheat 32.3 to 74.1 dw (**2.3**)	
Potato tubers (*Solanum* species)	61 *S. tuberosum* varieties 6 accessions (wild species)	52.1–137.3 dw (**2.6**)	Goyer and Navarrete (2007)
	12 individuals 4 varieties Multiple locations and seasons	80.9–118.7 dw (**1.5**)	
Potato tubers (*Solanum* species)	250 individuals 77 accessions (*S. tuberosum*) 10 accessions (wild species)	22.1–233.6 dw (**10.5**)	Robinson et al. (2015)
Chickpea seeds (*Cicer arietinum*)	4 varieties	351–589 (**1.7**)	Jha et al. (2015)
Lentil seeds (*Lens culinaris*)	4 varieties	136–182 (**1.3**)	
Pea seeds (*Pisum sativum*)	4 varieties	23–30 (**2.3**)	
Common bean seeds (*Phaseolus vulgaris*)	4 varieties	165–232 (**1.4**)	

(continued)

Table 6.2 (continued)

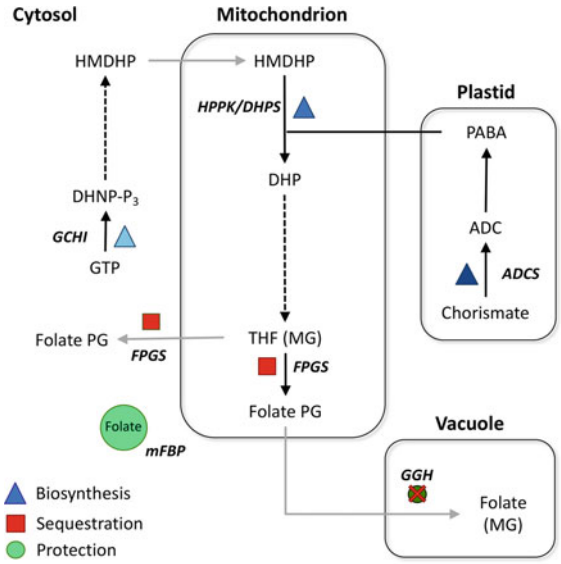
Crop	# Individuals screened	Folate contents μg 100 g^{-1} [a] (fold increase)	References
Common bean seeds (*Phaseolus vulgaris* L.)	11 individuals 4 varieties 6 F_1 hybrids 1 F_2 hybrid	217.2–338 (**1.6**)	Khanal et al. (2013)
Strawberry fruit (*Fragaria* × *ananassa*)	13 varieties	37–69 (**1.9**) year 1	Strålsjö et al. (2003)
		30–53 (**1.8**) year 2	
Tomato fruit (*Solanum lycopersicum*)	125 accessions (rep ripe fruit)	14–46 μg (**3.3**)	Upadhyaya et al. (2017)
	82 accessions (mature green fruit)	13–71 (**5.5**)	
Spinach leaves (*Spinacia oleracea*)	67 accessions	54.1–173.2 (**3.2**)	Shohag et al. (2011)

[a]When not defined, values were expressed in fresh weight basis; dw, dry weight

On the other hand, several folate engineering efforts have been reported over the past 15 years. These engineering works have also provided relevant knowledge about folate regulation in plants. Folate increments have been achieved by overexpressing biosynthetic genes and by sequestering and protecting folates from degradation within the cell (Fig. 6.7). These efforts are covered here by individual plant/crop.

6.4.1 Arabidopsis Leaves

This model plant was the first to be reported as engineered with the objective of boosting folates. GCHI from *Escherichia coli* (*folE* gene) was used to enhance the pteridine branch of the folate biosynthesis pathway (Hossain et al. 2004). Constitutive expression of *folE* elevated pteridines by up to 1250-fold, and folates incremented in a range of two- and fourfold. When the plant homolog (AtGCHI) was constitutively overexpressed in Arabidopsis, total pteridines increased up to 17.9-fold, and this increment did not significantly impact folate contents. PABA synthesis was also attempted in Arabidopsis, but expressing AtADCS in Arabidopsis was challenging; a slight overexpression could be achieved in $AtGCHI^+$ plants, but no impact in folate accumulation was observed. Moreover, when $AtGCHI^+$ plants were fed with PABA, folates were only slightly enhanced (Blancquaert et al. 2013b). Comparing the two works, it seems that to push folate production in Arabidopsis leaves, pteridines need to be greatly enhanced. Recently, another engineering attempt was conducted overexpressing the two committed steps of the biosynthetic pathway using genes from soy (Liang et al. 2019). Single overexpressors rendered increases in precursors;



Metabolic engineering achievements in enhancing folates in crops

Target	Engineering strategy	Folate accumulation (µg/100g - FW)	Reference
Rice seeds	WT	19	Storozhenko et al. 2007
	△	22	
	▲	8	
	△ + ▲	1723	
	△ + ▲ + ■	1840	Blancquaert et al. 2015
	△ + ▲ + ●	2530	
	△ + ▲ + ■ + ●	1750	
Potato tubers	WT	33	Blancquaert et al. 2013b
	△ + ▲	52	
	△ + ▲ + ▲	210	De Lepeleire et al. 2018
	△ + ▲ + ■	385	
	△ + ▲ + ▲ + ■	256	
Tomato fruit	WT	71	Diaz de la Garza et al. 2004
	△	178	
	▲	74	Diaz de la Garza et al. 2007
	△ + ▲	1133	
	WT	115	Akhtar et al. 2010
	◆	246	
Common bean seeds	WT	135	Ramírez Rivera et al. 2016
	△	325	
Maize seeds	WT	93*	Naqvi et al. 2009
	△	194*	
	Negative segregant	38*	Liang et al. 2019
	△ + ▲	158*	
Lettuce leaves	WT	35	Nunes et al. 2009
	△	189	
Wheat grains	WT	28*	Liang et al. 2019
	△ + ▲	65*	
	Negative segregant	24*	
	△ + ▲#	134*	

◀**Fig. 6.7** Strategies and achievements for folate engineering in crops. Biosynthetic steps are depicted in blue triangles; red squares denote folate sequestration within a compartment by elongation of the glutamyl tail by the folylpolyglutamate synthetase (FPGS); protection, green circles, was achieved by expressing a mammalian folate binding protein (mFBP) or downregulating deglutamylation. Engineered steps: **Biosynthesis**: GTP cyclohydrolase I, GCHI; hydroxymethyl dihydropterin pyrophosphokinase-dihydropteroate synthase, HPPK-DHPS; 4-amino-4-deoxychorismate synthase, ADCS. **Sequestration**: cytosolic or mitochondrial FPGS. **Protection**: downregulation of γ-glutamyl hydrolase, GGH; mammalian folate binding protein, mFBP. (**WT**) Wild-type control. Dotted lines denote several enzymatic steps. Gray lines indicate transport. Abbreviations are the same as those from Fig. 6.4. (*)Dry weight (#) codon-optimized genes driven by different promoters than first round

interestingly, the overexpression of each GmGCHI$^+$ isoenzyme gave different levels of pteridine accumulation, suggesting differences in enzyme activities. On the other hand, soy GmADCS$^+$ caused a modest twofold increase in PABA. Folates were not significantly changed in any of the lines. Once the two-gene strategy was applied, the results were similar to all previous attempts in Arabidopsis, folates accumulated slightly more (up to 1.9-fold) only when the high pteridine overexpressors were used. A detailed pteridine profile would shed more light on this apparent bottleneck in the folate synthesis for leaves, as the enhanced pteridines need to be biosynthetic, properly reduced, and within the mitochondria to synthesize more folates (Ramírez Rivera et al. 2016).

GGH expression was also engineered in Arabidopsis, and it was negatively correlated with folate accumulation (Akhtar et al. 2010). Downregulation of GGH by RNAi reduced the enzyme activity by 99%; this resulted in an increase of folate polyglutamylation, and folate contents were augmented by 34%. On the other hand, GGH overexpression caused a 40% loss of total folates, and the remaining folates had shorter PG tails. Downregulation of GGH activity is an option for increasing folate contents along with the other strategies discussed here. Similarly, 5-FCL Arabidopsis knockouts accumulated twice the amount of folates than WT leaves (Goyer et al. 2005). As previously mentioned, 5-FCL is the enzyme that puts back the 5-CHO-THF to the 1C donor pool (Fig. 6.5). 5-CHO-THF is the most stable reduced folate form (Fig. 6.1). It is not used in 1C reactions, but it is bioavailable as a vitamin for humans, as it can be absorbed; the 5-FCL mammalian homolog is methenyltetrahydrofolate synthetase (Anguera et al. 2003; Aufreiter et al. 2009). This strategy could represent a form of sequestering folates *in planta* if the plant growth and reproduction are not compromised.

6.4.2 Tomato Fruits

Folates were enhanced in tomato fruits by directing the overexpression of the two folate-committing steps specifically targeting ripening fruit, as previous biochemical data showed that the production of biosynthetic enzymes was shut down during fruit

ripening (discussed above in the biosynthesis section). E8 promoter is commonly used to drive fruit-specific transgene expression; it starts at the ripening process in climacteric fruit, at the ethylene production peak (Deikman et al. 1992). A mammalian (*Mus musculus*) GCHI was used (mGCHI) as, at the time, there was not yet any information about a possible feedback regulation for the plant homolog. As mammals do not synthesize folates, mGCHI starts the pteridine biosynthetic pathway for tetrahydrobiopterin (BH_4) production, which is an essential cofactor for phenylalanine hydroxylase (Heintz et al. 2013). This enzyme is inhibited by BH_4 through a feedback regulatory protein (Yoneyama and Hatakeyama 1998). Plants do not produce BH_4 and apparently have no homologs for the regulatory protein; therefore, the mammalian enzyme would not be inhibited by folates if expressed *in planta*.

mGCHI expression was correlated with high pteridine levels in ripening tomato fruit (Diaz de la Garza et al. 2004). Pteridine characterization uncovered previously unknown pteridine derivatives; neopterin and monapterin were found free and also conjugated as β-D-glucosides. Glycosylated pteridines might act as a storage form for these folate precursors. Total pteridines were enhanced from 3- to 140-fold, and HMDPT, the immediate precursor to form pteroate (Fig. 6.4), was the main pteridine of the pool. Folates were elevated up to twofold in the engineered lines, maintaining the same folate profile; 5-CH_3-THF hexaglutamylated was the main folate in tomato fruits.

The plastidial PABA branch was also enhanced in tomato fruits. Arabidopsis ADCS (AtADCS) was overexpressed in ripening tomatoes, causing increments of 19-fold on average (Diaz de la Garza et al. 2007). PABA overaccumulation alone was not sufficient to elevate folate levels in the fruit, confirming that pteridine production is limiting in the biosynthesis pathway. When $mGCHI^+$ segregants were crossed with $AtADCS^+$ lines, folates were boosted up to 25-fold, accumulating 840 μg/100 g of fresh fruit, which is more than the entire RDA for pregnant women. Folate profiles differed from the control fruit in the PG degree; this time, the majority of the folate pool was composed of monoglutamylated 5-CH_3-THF, showing that FPGS activity could not match folate overproduction. Surprisingly, $mGCHI^+/AtADCS^+$ fruits still accumulated significant amounts of precursors; both pteridines and PABA were enhanced to similar levels to those measured in a single overexpressors. Engineering both committed steps generated a bottleneck in the folate pathway. This limiting step was pinpointed to HMDHP transport into mitochondria, HMDP reduction status within the mitochondria, and/or HMDHP pyrophosphorylation by HPPK. Releasing this bottleneck has the potential to elevate the levels of the vitamin significantly higher.

The $mGCHI^+/AtADCS^+$ segregants were used for bioavailability studies in an animal model. Folate bioavailability changes depending on the folate derivative, PG degree, and food matrix (Clifford et al. 1991). Thus, it is relevant to show that the enhanced vitamin has the potential to elevate the folate status when consumed from the biofortified food matrix. A single-dose experiment was conducted in Winstar rats using tomato puree, and folates in the blood were measured over a 12 h period (Castorena-Torres et al. 2014). Equimolar amounts of 5-CH_3-THF and folic acid were given to the control group of animals. The differences in the processing and intestinal

absorption of folate forms are depicted in Fig. 6.3, which shows that quantifying folates in the bloodstream is a direct measurement of the immediate bioavailability of the folate consumed. The bioavailability of folates from biofortified tomatoes was equivalent to that from the synthetic 5-CH_3-THF MG, demonstrating that this biofortified crop has the potential to elevate folate status if consumed. Moreover, in this study folates were characterized from overripe fruit. This fruit was left up to 45 days after the breaker stage and it had a 40-fold increase on folate contents compared to the 25-fold found at 12 days (Diaz de la Garza et al. 2007; Castorena-Torres et al. 2014).

Engineering folates in tomatoes showed that: (a) folate biosynthesis continues when the fruit is ripe and aging, (b) all folate biosynthetic enzymes, both recombinant and endogenous, are present and active in the engineered senescent fruit, and (c) overaccumulated folates are stable within the fruit cell. These observations coincide with the transcriptome analysis conducted on engineered tomato fruits (Waller et al. 2009). Enhancing folate biosynthesis in ripening fruit did not change the fruit's transcriptome significantly; the transcripts of only 14 genes out of 11,000 screened increased significantly in the folate hyper accumulating fruit. From those, three genes downstream in the folate biosynthesis (ADCL, DHNA, and mitochondrial FPGS) were upregulated. Thus, enhancing the first committed steps in the folate biosynthesis triggered the expression of further genes in fruit tissue. All of these findings show that enhancing folates in fruit tissue has great potential to deliver folates for human nutrition if the fruits are consumed fresh; hence, losses from food processing do not apply for this plant food matrix.

6.4.3 Rice Endosperm

Extensive engineering work has been conducted on rice endosperm. The first round of engineering was conducted on the two-biosynthetic committed steps. AtGCHI and AtADCS were overexpressed individually and together under the regulation of the endosperm-specific globulin and glutelin B1 promoters, respectively (Storozhenko et al. 2007). AtGCHI alone increased the pteridine pool by 25-fold, and this increase did not cause an elevation of folate contents. The pteridine profile showed that neopterin was the main pteridine overproduced, while the immediate folate precursor HMDHP was not detectable. This result coincides with those obtained in Arabidopsis leaves and tomato fruits: Large increments of the pteridine pool are necessary to single-drive folate overaccumulation. PABA overaccumulation was elevated 49 times when compared to controls; however, unlike with tomato fruits, this accumulation caused a decline in folate pools in rice endosperm, hinting at a possible negative regulation loop.

Nonetheless, the double GCHI/ADCS overexpression caused similar effects in both plant organisms. A considerable increment of total MG folates was achieved (up to 1.72 mg/100 g FW). Cooked rice grains lost around 45% of the pool. However, even considering these losses, the amount of folate in this engineered cooked

rice met the RDA for an adult. Transcriptomic analyses were conducted on these engineered lines at different seed development times (Blancquaert et al. 2013a). Overall changes in gene expression were correlated with folate accumulation. A total of 235 genes were affected in their expression when considering all lines and times studied. The majority of them had unknown or putative functions. Three categories were the most affected: 1) stress/defense response and cell death; 2) protein mobility/modification and degradation; and 3) cell cycle, structure, organization, and development genes. Interestingly, none of the genes affected belonged to folate biosynthesis or direct folate interconversion. Yet, there were a few genes in which variations in gene expression could be attributed to changes in the folate levels and their precursors.

The bioavailability of folates from this biofortified rice was evaluated in rats. The study was conducted for a 12-week period in which engineered, non-engineered, folate-free, and folic acid-fortified rice diets were fed to folate-depleted animals. Almost all markers measured (e.g., plasma folates, homocysteine, hematocrit, etc.) were comparable to the diet fortified with folic acid, while folate-depleted and non-engineered rice-fed animals showed all of the negative consequences of a lack of folate nutrition (Kiekens et al. 2015).

The second round of folate engineering in rice was done by targeting folate sequestration within cell compartments and protecting them via a mammalian folate binding protein (FBP) (Blancquaert et al. 2015). Enhanced folates in rice declined during seed storage, losing 50% of the vitamin levels after 4 months and 60% after 8 months. This, plus the losses observed during cooking, prompted targeting folate stabilization by engineering. Folate sequestration within the cell was explored by overexpressing FPGS isoforms, targeted to either the mitochondria or cytosol. Folate protection was attempted by expressing a modified mammalian FBP found in bovine milk. The original bovine gene was codon-optimized and modified. First, the signal for membrane anchorage was removed, and second, three recombinant versions were generated: the truncated soluble version alone (sFBP), truncated version fused with a β-carbonic anhydrase 2 gene from Arabidopsis (CAFBP), and another fusion protein with rice glutelin B4 (GluB4FBP) to solubilize and stabilize this original transmembranal folate receptor.

All these genes were overexpressed in rice endosperm from plants already engineered for pteridines and PABA. All possible single and double combinations were tested for folate increments and stability. Cytosolic FPGS plus FBP gave an immediate folate boost in rice endosperm up to 2.5 mg/100 g FW, double what was initially reported by the two-gene overexpression strategy. By extending PG tails of folates in the cytosol and adding the protection of FBP, folates were sequestered within this compartment, and a possible feedback inhibition within the mitochondria could be released. These folates were stable in rice seeds stored for 4 months at 28 °C. Moreover, the enhanced phenotype was maintained throughout generations. Interestingly, this approach caused an upregulation of the endogenous GGH genes, the expression of which was positively correlated with that of recombinant FPGS. This second round of engineering achieved a massive folate accumulation in rice endosperm that was stable throughout storage.

Further folate bioavailability studies in cooked biofortified rice could determine if: (a) protected folates are more stable during the cooking process, (b) folates can be released from protection by the thermal treatment, or (c) these folate forms are more or less bioavailable than those with more MG percentage and non-protein bound. Even if the resulting bioavailability was revealed to be inferior to that of non-protected folates in rice seeds, the amount of stable folates achieved by this approach would likely still be able to increase folate status if consumed.

There have been other attempts to engineer folates in rice. Eight biosynthetic genes from Arabidopsis were constitutively overexpressed individually, and the progeny of selected crosses were generated (Dong et al. 2014). GCHI, ADCS, and FPGS caused folate increases, with GCHI being the most effective (up to 6.1-fold). However, when GCHI plants were individually crossed with ADCS, DHNA, HPPK, DHFR, DHFS, and FPGS, folates did not increase compared to the parental GCHI. Neither precursors nor enzyme activities were reported in this work that could allow for a better assessment of the engineering results.

In another study, endogenous FPGSs, *Bos taurus* FBP, and *Rattus norvegicus* glycine *N*-methyltransferase (GNMT) were individually overexpressed in rice (Abilgos Ramos 2010). All engineered lines presented increases in total folate contents, and GNMT, a mammalian enzyme that is inhibited by two molecules of 5-CH_3-THF pentaglutamate (Luka et al. 2007), caused the highest accumulation of around eight-fold. Interestingly, both FBP and GNMT rice plants were reported to produce only around 20 and 50%, respectively, of the seeds produced by a non-transformed plant, suggesting that sequestering folates by protein binding can have negative effects on plant development. However, an effect on seed yield was not reported when modified forms of bovine FBP were engineered (Blancquaert et al. 2015).

6.4.4 Potato Tubers

Biofortification of potato tubers with folates has been difficult to achieve. The two-biosynthetic gene strategy has not worked for this plant organ as it has for tomato fruits and rice endosperm. AtGCHI and AtADCS controlled by the tuber-specific patatin promoter yielded up to threefold increases in folates, while pteridines and PABA levels remained high. Nonetheless, the increase observed in young tubers was lost in mature tubers (Blancquaert et al. 2013b). The presence of engineered precursors in potato tubers suggests bottlenecks in the biosynthetic pathway, which were not limited in previous engineering works. This was confirmed by the second round of engineering, when the overexpression of mitochondrial Arabidopsis FPGS (mtAtFPGS) and/or OsHPPK/DHPS rice, along with AtGCHI and AtADCS, was necessary to drive a steady 12-fold increases of folates in potato tubers (De Lepeleire et al. 2018). In addition, folates were stable during storage for 9 months at 4 °C. Two AtGCHI/AtADCS backgrounds were used for re-transformation expressing OsHPPK/DHPS and mtAtFPGS, individually and combined. All three combinations rendered similar folate levels, and, interestingly, each AtGCHI/AtADCS background

presented a depletion of either biosynthetic pteridines or PABA. Thus, in the case of potato tubers, pteridines and PABA can still be limited, despite the overexpression of the committed steps for their production.

Folates enhanced in this work were, in all cases, equally or more polyglutamylated than those from WT tubers, even for those lines where mtFPGS was not engineered. The amount of folates accumulated in this case was up to 180 μg/100 g FW. This number is ≈10 times lower than rice and 5 times lower than tomatoes. The PG profile of folates varies among plants, and it is the result of the FPGS and GGH isoforms, the activities of which could also vary among them (Ramos-Parra et al. 2013). Endogenous potato FPGS was able to polyglutamylate folates at this engineered flux; therefore, further increases in the synthesis of folates may also change PG profiles for this organism.

6.4.5 Common Bean Seeds

Contrary to rice, tomatoes, and potato tubers, legume seeds are well known for their high folate contents (USDA 2018). Folate engineering in common beans was attempted by AtGCHI expression controlled by the seed-specific β-conglycinin promoter. Three pinto varieties were used as background (Ramírez Rivera et al. 2016). As with other engineering examples, this overexpression caused a massive pteridine build-up that drove enhanced folate production up to 3.3-fold. As WT folate contents were relatively high, this increment signified a folate accumulation of up to 325 μg/100 g FW, which would represent 82% of the RDA. Dried WT common bean seeds accumulate a fair amount of pteridines; however, the majority of them are pteridine- or folate-breakdown products, rather than those that participate in folate biosynthesis. AtGCHI overexpression caused a hyper-accumulation of biosynthetic pteridines of up to 148-fold, while the oxidized breakdown products increased in significantly lesser amounts.

Surprisingly, all engineered lines overproduced PABA, even though this branch was not engineered. Endogenous ADCS transcription seemed to be feed-forward upregulated; this is the only case that has shown this effect when pteridines were engineered. Despite this endogenous upregulation, the folate boost was modest. PABA pools in WT common bean seeds are mostly present as free PABA, unlike with other plants (Quinlivan et al. 2003). PABA pools in engineered seeds had a higher proportion of esterified PABA in some cases, but the majority of them accumulated about half the pool as free PABA. Thus, there is a bottleneck in the folate biosynthetic pathway in common bean grains, as both precursors were present. Nevertheless, PABA fed to AtGCHI seeds after harvest by imbibition caused slight, but significant, folate increments. All measurements were conducted in desiccated bean seeds, and no data was provided about how these pools behave during seed development when the promoter begins acting. The engineering works discussed here demonstrate that a concerted production of biosynthetic pteridines and PABA need to occur within the cell to boost folate synthesis. Thus, PABA engineering at the same time

as pteridines will likely enhance the folate levels achieved by engineering only the pteridine branch.

6.4.6 Maize kernels and Lettuce leaves

Corn was biofortified with three vitamins by stacking several genes controlled by seed-specific promoters (Naqvi et al. 2009). Vitamin C (ascorbate) was increased sixfold by the expression of rice dehydroascorbate reductase; total carotenoids were boosted 112-fold by the expression of maize phytoene synthase and carotene desaturase from *P. ananatis* (*crtI* gene). *E. coli* GCHI was used to increase folates, which, as with other crops, were enhanced up to twofold. No measurements of precursors were provided in this work. Because *E. coli* GCHI caused a massive pteridine production in Arabidopsis leaves (Hossain et al. 2004), it is probable that it has similar effects on corn seeds. Similarly, lettuce folate levels were increased by 8.5-fold by overexpressing a GCHI from chickens (*Gallus gallus*). Lettuce leaves accumulated 188 μg/100 g FW. The coexpression of soy Gm8gGCHI and GmADCS was also applied to maize; gene expression was driven by endosperm-specific promoters (Liang et al. 2019). Dried kernels accumulated between 3–4.2 times greater folate contents than controls. Adding ADCS caused a further slight increase in folates; however, PABA production was not high enough, pteridines still over accumulated while PABA was limiting in double overexpressors. Boosting PABA levels along with the overexpression of more genes from the biosynthetic pathway, will probably generate higher folate increases in this crop.

6.4.7 Wheat grains

Wheat grains (*Triticum aestivum* cv. Fielder) have been subjected to two rounds of engineering (Liang et al. 2019). In the first round, both soy proteins used in Arabidopsis and maize (Gm8gGCHI and GmADCS) were coexpressed in wheat endosperm. This strategy doubled in average folate contents and, contrary to maize, PABA was over accumulated in higher amounts than pteridines in all the engineered grains, suggesting differences in folate synthesis regulation between the two cereal grains. The second round of engineering consisted of using codon-optimized genes from soy (Gm8gGCHI) and this time ADCS from tomato. Additionally, the endosperm specific promoters were changed; while in the first-round maize and rice promoters were used, the second round used the wheat glutenin 1DX5 promoter. All these changes together caused higher pteridine accumulation, while PABA overproduction seemed enhanced in similar amounts than the previous round. The enhancement of both precursors further elevated folate contents up to 5.6 times more than negative segregants. However, precursors were still in higher amounts than controls in double transformants (both less than an order of magnitude) hinting to the presence of a

bottleneck in the pathway present in wheat grains. 5-CH_3-THF was the folate that accumulated the most in the engineered lines followed by 5-CHO-THF. It is interesting to note that each cereal grain (rice, maize, and wheat) that has been biofortified with the two-committed steps strategy has accumulated precursors with different tendencies, rendering particular folate accumulations (Fig. 6.7). Folate biofortification strategies, thus, need to be adjusted to each tissue and plant species.

6.5 Socioeconomic Studies and Potential of Biofortification with Folates

Rice, maize, potatoes, and legumes are staple crops consumed around the globe by millions of people who live in poverty. Tomatoes are the most consumed fruit worldwide (FAO stat 2014). Enhancing folates in these targeted crops has the potential to increase folate status in many parts of the world, as these crops are part of the basic diet for many populations, and in poor areas, they are cultivated for self-consumption. Economically, implementing the release of biofortified varieties can also be feasible. It has been estimated that biofortification costs would be a fraction of those needed for vitamin supplementation (Mayer et al. 2008). However, genetically modified crops have a current general negative perception by the public; this has contributed to stalling the liberation of Golden Rice, the provitamin A-biofortified variety and the most advanced biofortified crop by metabolic engineering (Moghissi et al. 2016). In an attempt to discover elements, that can show their potential implementations, socioeconomic studies have been conducted on biofortified rice with folates. Cost-effectiveness was calculated assuming biofortified rice was implemented in China, specifically in a region with a very high risk of folate deficiency. In addition, willing to pay (WTP) premiums were also calculated in the same region based on consumer preferences between folic acid supplementation versus folate biofortification. The calculated data showed that biofortified rice has the potential to highly improve health with moderate cost-effectiveness (De Steur et al. 2012). In addition, WTP premiums would be higher for biofortified rice than for folic acid pills, as people would prefer to consume folates from the staple crop rather than taking supplement pills (De Steur et al. 2013). These studies show that people can have positive reactions if they are informed about the benefits of biofortified plant foods, confirming the importance of public information. Moreover, a recent systematic review and meta-analysis work covering several biofortified food crops also indicated that biofortified crops by engineering could be cost-effective and accepted by the public (De Steur et al. 2017).

More studies need to be conducted to advance biofortified lines to commercial varieties. Toxicology studies need to be assessed, as many of the biofortified lines still accumulate folate precursors. Humans produce pterins, and PABA has been declared as Generally Recognized as Safe (GRAS) by the FDA (Hoagland 1950); however, humans are not exposed to high levels of HMDHP, the immediate folate precursor

that, in some cases, remains accumulated in the proof-of-concept lines. These studies are also needed because these are genetically modified varieties. On the other hand, field trials are also part of the pipeline to test and generate a commercial variety that has the same or better yields than the current ones. More bioavailability studies, first with animal models, then as clinical trials after toxicity clearance, will also support the nutritional benefits that folate-enhanced crops will have for elevating folate status. In addition, studies about the regulation of folate metabolism are needed, as nothing is known about the transcriptional regulation of folate biosynthetic or salvage genes. Understanding regulatory *cis* elements that affect folate accumulation will provide strategies for genome editing using CRISPR-Cas techniques. Improving crops by genome edition has great potential for engineering quantitative traits more quickly than conventional breeding and genetic modification, with the potential of having a better public acceptance than recombinant expression (Rodríguez-Leal et al. 2017; Scheben and Edwards 2017).

Years of research and trials are awaiting; however, this work is extremely relevant as micronutrient deficiency, also known as hidden hunger, is a global public health problem. Folate deficiency elevates the risk of human diseases for all ages. Population growth and climate change have made it urgent for scientists to come up with alternatives to feed and nourish populations at risk. Based on the works covered here, it is safe to assess that the ultimate goal, to provide a whole adult RDA of folates in one portion of a cooked plant food, is achievable. A biofortified crop with folates can also be crossed and complemented with other micronutrients (e.g., iron, zinc, provitamin A, etc.), generating a nutrient-dense plant food. All of these studies enhancing folates have contributed to increased knowledge about folate metabolism and its role in plant physiology; more knowledge will also potentially help to improve plant fitness and yields in the future.

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Chapter 7
Thiamine and Its Role in Protection Against Stress in Plants (Enhancement in Thiamine Content for Nutritional Quality Improvement)

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Abstract Thiamine or vitamin B_1 plays an indispensable role in many metabolic reactions. The active form, thiamine pyrophosphate (TPP), functions as a cofactor in various crucial metabolic reactions including glycolysis, pentose phosphate pathway, and the tricarboxylic acid cycle in all living organisms. Recently, thiamine is also associated with the induction of systemic acquired resistance (SAR) in the plant. It has also been shown that thiamine has a role in boosting plants' immunity and defence system. Many plants have been investigated and indeed thiamine may be one of the key molecules involved in plant protection against stress. Numerous studies have shown that the expression of thiamine biosynthesis genes was upregulated upon both biotic and abiotic stress induction in various plants. Various analyses including looking at the expression of thiamine biosynthesis genes, the accumulation of thiamine and its intermediates and also on enzyme function complementation studies have supported the role that thiamine may play in plant protection. In this chapter, the role of thiamine as a stress-responsive signalling molecule, its biosynthesis pathway and how it is being regulated will be discussed. The application that entails the understanding of this role will be briefly described and hence provide the support for the suggestion of its role in protection against stress in plants.

Keywords Thiamine · Vitamin B_1 · Stress · Plant protection

7.1 Introduction

Plants are sessile organisms that are inevitably exposed to unfavourable biotic and abiotic stresses throughout their lifetime. In terms of crop plants, stresses which include climate and environmental changes and attack by pathogens can severely hamper the productivity of these plants. The current research is accelerating towards finding ways to control the effects of both biotic and abiotic stresses in plants, in a

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more organic and environmental-friendly manner. Recently, the studies of plant and microorganism interactions or also known as biocontrol agents have been of interest since these interactions actually play a role in alleviating stresses in the host plants (Boivin et al. 2016). On top of that, a 'feedback regulation' mechanism of stress whereby the stress itself can actually induce plant tolerance and even resistance has been demonstrated. The discovery of an additional role for a common vitamin which has been known to be functional as a cofactor has now been described (Goyer 2010). Thiamine or also known as vitamin B_1 is an enzymatic cofactor in metabolic reactions have now also been known to be involved in plant adaptation and alleviation of biotic and abiotic stresses (Rapala-Kozik et al. 2008; Tunc-Ozdemir et al. 2009). It was observed that there was an accumulation of thiamine when the plants were subjected to salinity stress, oxidative stress, and pathogenic attack (Rapala-Kozik et al. 2008; Tunc-Ozdemir et al. 2009; Zhou et al. 2013; Balia Yusof et al. 2015; Kamarudin et al. 2017a). It is now understood that thiamine formed an indirect role in enhancing anti-oxidative capacity in the plants, which is important in defence responses (Zhou et al. 2013). Yet, the exact mechanism of biosynthesis of thiamine in response to stresses is still poorly understood.

7.2 Thiamine

Thiamine or vitamin B_1 is one of the first B vitamins discovered in 1910 in Japan by Umetaro Suzuki (Goyer 2010). The structure composed of a pyrimidine ring joined with a thiazole moiety. The active form, thiamine pyrophosphate (TPP), is an important cofactor in various metabolic reactions. TPP is a cofactor of pyruvate dehydrogenase which participates in the oxidative decarboxylation of pyruvate to acetyl-CoA, synthesis of amino acid such as valine, leucine and isoleucine, 2-oxoglutarate dehydrogenase which is involved in Krebs cycle (Goyer 2010; Du et al. 2011; Goyer and Haynes 2011). TPP is also a cofactor for transketolase in the oxidative pentose phosphate pathway. Downstream biochemical reactions of Krebs cycle are the oxidative phosphorylation pathway where the electron transport chain is generated to synthesize ATP, which is important in energy production in all living organisms. In human nutrition, thiamine is one of the essential micronutrients. Since thiamine and its ester forms are only synthesized in plants and microbes, therefore, animals and humans need to obtain thiamine exogenously from the diet.

7.3 The Thiamine Biosynthesis Pathway

The thiamine biosynthesis pathway in plants shares similarity with the ones described in bacteria and yeast (Begley et al. 1999; Li et al. 2010). Table 7.1 is a summary of the inter-changeable terms of the thiamine biosynthesis enzymes and the homologs of different plants and algae. As shown in Fig. 7.1, the pathway starts from the

Table 7.1 Biochemical functions of thiamine biosynthesis enzymes

Abbreviation	E.C number	Homologs	Biochemical function
THIC	4.1.99.17	*THIC* in rice, *Arabidopsis*, *Chlamydomonas reinhardtii*, *Anabaena* sp. and maize. THI5 in yeast	Hydroxymethylpyrimidine phosphate synthase
THI1/THI4	2.8.1.10	*THI1* in rice and *Arabidopsis*, *THI4* in yeast, *C. reinhardtii* and maize, *THIG* in *Anabaena* sp.	Thiazole biosynthetic protein
TH1	2.5.1.3	*TH1* in rice, *Arabidopsis*, and maize. *THI6* in yeast. *THID/THIE* in *C. reinhardtii* and *Anabaena* sp.	Hydroxymethylpyrimidine phosphate kinase
TH2	3.1.3.100	*TH2* in *Arabidopsis*	Thiamine monophosphate phosphatase
TPK	2.7.6.2	*TPK* in rice, *Arabidopsis*, *C. reinhardtii* and maize. *THI*80 in yeast, *THIL* in *Anabaena* sp.	Thiamine pyrophosphate kinase

precursor 5-aminoimidazole ribonucleotide (AIR) to the pyrimidine moiety of thiamine, which is hydroxymethylpyrimidine phosphate by the enzyme encoded as *THIC* (HMP-synthase). The second parallel pathway stems from NAD, glycine, and S-donor to form the thiazole moiety of thiamine, hydroxyethylthiazole phosphate which is synthesized by the enzyme hydroxyethylphosphate synthase (*THI4*). Both thiazole and pyrimidine moiety are joined together by the enzyme *TH1* (thiamine bifunctional enzyme) to form thiamine monophosphate. Thiamine monophosphate is dephosphorylated by a phosphatase known as thiamine monophosphate phosphatase (*TH2*) to form thiamine (Mimura et al. 2016). All the reactions occur in the chloroplast. The last step is the phosphorylation of thiamine to its active form, thiamine pyrophosphate (TPP) by the enzyme thiamine pyrophosphokinase (*TPK*) that occurs in the cytosol. TPP only functions in the mitochondria; therefore to be biologically active, TPP must be transported into the chloroplast or mitochondria (Goyer 2010).

The transcripts of *THI4*, *THIC*, *TH1* and *TPK* were found in leaves, roots and stems. Interestingly, the expressions of the transcripts are the highest in the leaves which corroborates the fact that thiamine biosynthesis occurred in the chloroplasts. It was noted that thiamine biosynthesis is differentially regulated depending on plant tissues (Goyer 2010). Preliminary study on transcripts of *THI4* and *THIC* has shown that it was not amplified in root tissues of oil palm seedlings (Wong et al. 2016).

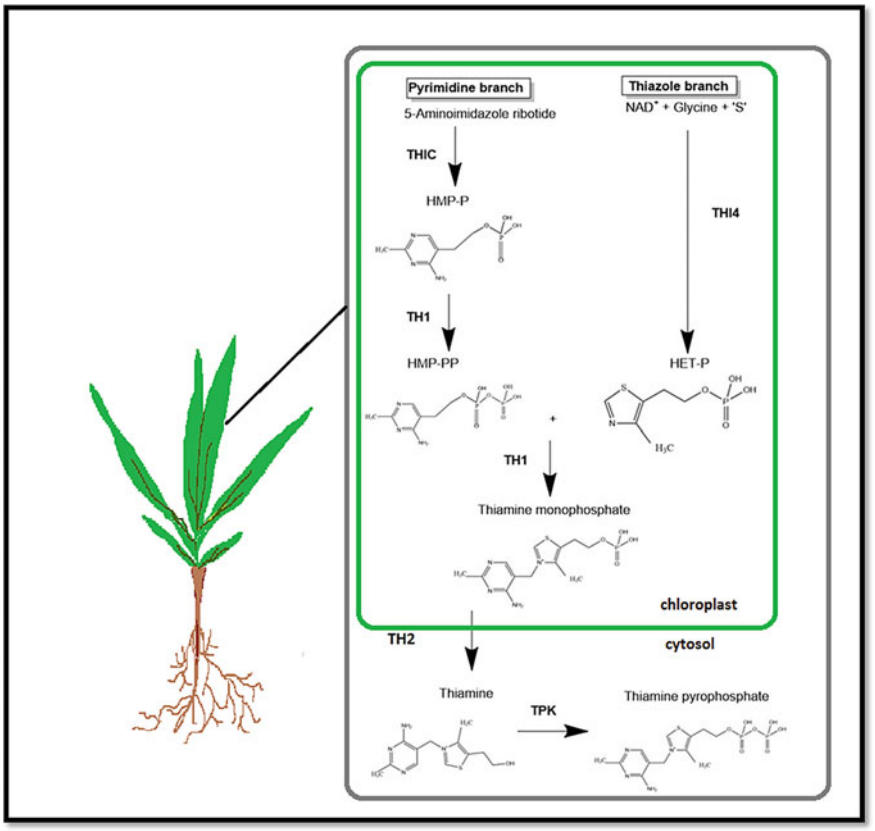

Fig. 7.1 Thiamine biosynthesis pathway in plants. HMP-P is hydroxymethylpyrimidine phosphate, HMP-PP is hydroxymethylpyrimidine pyrophosphate, HET-P is hydroxyethylthiazole phosphate. *THIC* is hydroxymethylpyrimidine-synthase, *THI4* is thiazole biosynthetic protein, *TH1* is hydroxymethylpyrimidine phosphate kinase, *TH2* is thiamine monophosphate phosphatase and *TPK* is thiamine pyrophosphate kinase. Reproduced from Kamarudin et al. (2017a)

7.4 Role of Thiamine in Biotic and Abiotic Stresses in Plants

Vitamins have been used as a control agent for different diseases (Ahn et al. 2005; Palacios et al. 2014). In plants, thiamine treatment has been shown to adverse pathogenic infections. For example, thiamine treatment in soybean enhances resistance to charcoal rot disease (Abdel-Monaim 2011), rice against sheath blight disease (Bahuguna et al. 2012), and grapevine against *Plasmopara viticola* (Boubakri et al. 2012). The mechanism of disease suppression through application of thiamine is explained by the activation of a plethora of host defence responses. In *Arabidopsis thaliana*, thiamine treatment activates pathogenesis-related protein (PR-1) and

phenylalanine lyase (PAL). In addition, thiamine treatment in grapevine reduced downy mildew development compared to untreated control in a dose-dependent manner by inducing hydrogen peroxide generation, callose disposition and host resistance (HR) cell death. Similarly, thiamine treatment successfully controlled charcoal rot disease in soybean plants by the induction of defence-related enzymes including peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and pathogenesis-related (PR) chitinase (Abdel-Monaim 2011). Phytohormones are also involved, as a study has indicated that two genes known to be regulated by salicylic acid were upregulated and while gene regulated by methyl jasmonate was downregulated in thiamine-treated barley peas after aphid infestation (Hamada and Jonsson 2013). Therefore, thiamine treatment primed plants from pathogen attack due to the enhanced activity of the induced systemic resistance via induction of defence-related enzymes, PR proteins, and phytohormones.

As highlighted earlier, besides its role as a cofactor in the primary metabolism, thiamine has been shown to play a role in plant adaptation against stresses. Several studies have documented the modulation of the expression of thiamine biosynthesis genes when the plants were subjected to a variety of stresses (Rapala-Kozik et al. 2008; Tunc-Ozdemir et al. 2009; Rapala-Kozik et al. 2012).

Notably, Tunc-Ozdemir et al. (2009) asserted the role of TPP as important stress response molecule as it was demonstrated that thiamine biosynthesis is induced in *Arabidopsis* in response to different abiotic stresses. Relative to control seedlings, total thiamine content was significantly induced in *Arabidopsis* seedlings when subjected to cold, osmotic, and salinity conditions. The increase in total thiamine content which is the sum of thiamine, thiamine monophosphate (TMP), and thiamine pyrophosphate (TPP) was due to the large increase of TPP, which is the active form of thiamine. Transcript abundance of the *THI4*, *THIC*, *TH1* and *TPK* was significantly increased when subjected to paraquat stress, where the largest increase in transcript abundance was found in TPK gene (sixfold). Similarly, at increasing the concentration of PEG and NaCl to induce osmotic and salt stress, respectively, total thiamine content in maize seedlings was significantly increased. The increase in total thiamine content correlated with the gain in abscisic acid (ABA) content in maize seedlings (Tunc-Ozdemir et al. 2009). In addition, under drought and salinity stress conditions, a moderate increase in transketolase activity, which is the major TPP-dependent enzyme, was also detectable. The perturbation in transketolase activity suggests the role of thiamine metabolism in plant adaptation to stresses (Tunc-Ozdemir et al. 2009).

In oil palm, which is the primary commodity in Malaysia, it is interesting to examine the responses to biotic and abiotic stresses, specifically on the expression of thiamine biosynthesis genes. A semi-quantitative reverse transcriptase PCR was performed and showed that *THI1*/*THI4* and *THIC* were upregulated when the oil palm seedlings subjected to *Ganoderma boninense* infection (Balia Yusof et al. 2015), and also oxidative, salinity and osmotic stresses (Wong et al. 2016; Abidin et al. 2016). Wong et al. (2016) reported that the increase in transcript level of *THI1*/*THI4* and *THIC* were detectable at early stages of day 3 of PEG-induced osmotic stress in oil palm seedlings. On the other hand, at subsequent days of day 7 and day 30, the

decrease in level of expression of THI1/THI4 and THIC suggests that the seedlings have adapted to stress. It is worth noting the different levels of increment of expression of *THI1/THI4* and *THIC* when the oil palm seedlings were subjected to stresses, where it was shown that the increase in the level of expression of *THI1/THI4* was higher than *THIC*. Overall, it was demonstrated that the expression of *THI1/THI4* and *THIC* were upregulated with an increase in the concentration of the stress inducer (Abidin et al. 2016).

The efforts of **biofortification of thiamine** are currently focused on the rice. Wang et al. (2006) reported that transgenic rice that has a knocked-out *OsDR8* gene which has a high-sequence similarity to maize genes *THI1/THI4* showed reduced resistance to bacterial blight and sheath disease caused by *Xanthomonas oryzae* and *Magnaporthe grisea* (Fig. 7.2). It is suggested that biofortification of thiamine through genetic engineering will lead to higher resistance against bacterial blast diseases and also increasing nutritional bioavailability (Dong et al. 2015). However, overexpression of *THIC* or *THI1/THI4* in rice lines did not result in an increase thiamine level in rice seeds. Interestingly, when both *THIC* and *THI1/THI4* were overexpressed, thiamine accumulation was observed up to fivefold in unpolished seeds. However, overexpressed *THIC* and *THI1/THI4* lines did not show enhanced resistance towards *X. oryzae* (Dong et al. 2016). This implies that thiamine accumulation does not necessarily enhance resistance towards diseases and the relationship between thiamine metabolism and disease resistance is still poorly understood (Dong et al. 2016).

Fig. 7.2 Transgenic rice that has a knocked-out OsDR8 gene which is homologs of *THI1/THI4* showed an apparent disease symptoms caused by *Xanthomonas oryzae* and *Magnaporthe grisea*. Adapted from Wang et al. (2006)

7.5 Role of Thiamine in Plant Growth

Thiamine is also important for the development and growth of plants. Previous studies have shown that thiamine is indispensable for in vitro growth and development of excised roots in many plants (Palanisamy et al. 1998). A maize thiamine auxotroph exhibited a defect in shoot meristem growth (Woodward et al. 2010). Apart from that, thiamine is used as growth regulators in vegetative propagation of *Jatropha curcas* (Dhillon et al. 2011) and embryogenic callus induction in *Zoysia japonica* turgrasses (Asano et al. 1996). Recently, thiamine pre-treatment in soybean seeds resulted in better growth as indicated as higher nodules per plant, and fresh and dry weight per plant compared with control (Abdel-Monaim 2011).

7.6 Enhancement of Thiamine Content in Plants

As described earlier, thiamine has shown as to act as a cofactor and a signalling molecule to alleviate plant stress and enhance disease resistance. Previous studies have shown that supplementation and accumulation of thiamine in plants showed no evidence of toxicity towards the plants (Pourcel et al. 2013). Thiamine production has also been shown to be regulated in order to balance the demand and supply of the vitamin. Other than that, thiamine biosynthesis is regulated via riboswitch-dependent gene regulation and tissue specificity, stress dependence, and post-translational regulation (Belanger et al. 1995; Ribeiro et al. 1996; Papini-Terzi et al. 2003).

It is generally understood that the total thiamine content in plants is composed of thiamine, thiamine monophosphate (ThMP) and thiamine diphosphate (ThDP) (Goyer 2010). Studies have been done in the overexpression of THIC and THI4 simultaneously where it has shown to increase the level of thiamine to approximately sixfold and ThDP level to approximately twofold. As compared to a single overexpression of either THIC or THI4, the study showed no elevation of total thiamine content (Dong et al. 2016). This clearly shows the correlation between thiamine biosynthesis genes and thiamine production. This finding suggested that the fortification of thiamine in plants may be achievable via thorough an understanding of the gene expression of the enzymes involved in the thiamine biosynthesis pathway. Due to that, the studies on the effects of biotic and abiotic stress towards the elevation of the expression of thiamine biosynthesis genes have been done widely (Balia Yusof et al. 2015; Wong et al. 2016; Abidin et al. 2016; Kamarudin et al. 2017a, b). The idea of utilizing stress in order to elevate the biosynthesis of thiamine in plants may contribute to the positive achievement in fortifying thiamine in crops.

The other obvious method is clearly through genetic manipulation. It has been suggested that thiamine fortification in plants may be possible via genetic engineering. A previous study on Arabidopsis proved that the mutation of the well-known regulatory element involved in thiamine biosynthesis pathway, the riboswitch has produced an Arabidopsis mutant with impaired TPP riboswitch activity, and hence, the plant

has an enhanced accumulation of total thiamine and its derivatives (Bocobza et al. 2013). However, more CO_2 production was observed in this mutant due to increasing TPP concentrations. High concentrations of TPP have led to an increased metabolic flux into the TCA cycle and pentose phosphate cycle, and these conditions can cause a significant increase in the organism respiratory rate (Bocobza et al. 2013). Apart from that, overexpression THIC and THI4 genes in Arabidopsis and rice have shown to increase thiamine and ThDP levels of up to sixfold and twofold, respectively, in Arabidopsis and an increase of total thiamine level in rice of up by fivefold (Dong et al. 2015, 2016). On top of that, genetic manipulation of another enzyme in thiamine biosynthesis pathway TPK in Arabidopsis caused an increased expression of TPK of up to 30-fold besides an elevation in transketolase enzyme activity by 2.5 fold (Khozaei et al. 2015). However, the mutant plant somehow showed some chlorosis and also has been observed to be slow-growing. These are somehow the expected consequences due to genetic manipulation procedure which may be avoided.

7.7 Conclusion

Therefore, in general, the literature here clearly suggested that thiamine may be involved in stress protection in plants. The gain in thiamine content will be beneficial to the plant as numerous studies have shown that thiamine is associated with induced systemic resistance that involves the production of NADPH, ROS and other defence metabolites. Taken together, it implies that thiamine may augment disease resistance ability through enhancement of thiamine biosynthesis. The identification of thiamine biosynthesis genes in plants will enable us to unravel other possible roles of thiamine in abiotic and biotic stress responses. The overexpression of thiamine may produce plants with enhanced resistance to diseases and stresses. Therefore, the understanding of the role of thiamine in plant protection could be adapted as disease resistance strategies in the framework of sustainable agricultural practices.

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Chapter 8
Vitamins B_6-, C-, and E-Enriched Crops

Manish Sainger, Darshna Chaudhary, Ranjana Jaiwal, Anil K. Chhillar and Pawan Kumar Jaiwal

Abstract Bourgeoning population and global climate change have put a tough challenge of feeding a large number of undernourished (with insufficient calorie intake) and malnourished (with limited or no access to essential micronutrients, vitamins, and minerals, causing the so-called hidden hunger) people globally. During the last few decades, the increase in production of calorie-rich staple food crops has resulted in a decrease in the number of undernourished people from over 1 billion to less than 800 million. However, no such equivalent increase in the production of non-staple foods (pulses, vegetables, fruits, and animal products) has been seen. The micronutrient malnutrition is still affecting more than 2 billion people or one-in-three people globally. Further, staple food crops are poor in vitamins that are further lost during storage, processing, and cooking. Vitamin deficiencies are prevalent in people who are solely dependent on staple crops for their diet and cannot afford diversified diet and have limited access to supplementation (multivitamin pills) or fortified food (addition of vitamins to food). Vitamin deficiencies in human cause severe physical and mental damages and are associated with enormous economic losses. Biofortification is a cost-effective and sustainable alternative to enhance vitamins in edible parts of the plant through breeding or metabolic engineering. The present chapter focuses on three relevant vitamins, B_6, C, and E. An overview of their role in plants, metabolism, rational behind biofortification, and advances in manipulation of their contents in plants by the maker-assisted selection and metabolic engineering is presented.

Keywords Vitamins · Metabolism · Biofortification · Vitamin B_6 · Ascorbate · Tocochromanols

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8.1 Introduction

Vitamins are the vital organic compounds required by humans and animals in small amounts (micronutrient) for their normal growth and development. They act as cofactors of metabolic enzymes. These compounds are synthesized by bacteria, fungi, and plants but not by humans and animals which are dependent on a balanced or varied diet for their supply. However, most of the people in developing countries are dependent on monotonous diet of the staple food crops like rice, wheat, maize, potato, and cassava that contain micronutrients in small amounts. A proportion of these nutrients is lost during food processing, preservation, or cooking and thus fails to meet the natural daily human body requirements, resulting into their deficiencies which subsequently lead to health disorders/diseases. Total 13 vitamins are broadly grouped into water soluble (B and C groups) and fat soluble (A, D, E, and K). Their demand in food, feed, cosmetic, and pharmaceutical industries has increased leading to the expansion of their global market volume from 5 to 75% during 1999–2012 (Schwechheimer et al. 2016). Currently, their market demand is met by their chemical synthesis which is unsustainable (Vandamme and Revuelta 2016). Recent developments in genomics, high marker density maps, and genetic resources have improved our understanding of vitamin biosynthesis pathways and their regulation to increase their contents in edible parts of plants using plant breeding (marker-assisted breeding and QTL) and transgenic (metabolic engineering) techniques (Strobbe and Van Der Straeten 2018; Smirnoff 2018; Mène-Saffrané 2018). The current status and future directions for the biofortification of vitamin B_6, C, and E in model and crop plants are discussed in the present chapter.

8.2 Vitamin B_6

Vitamin B_6 belongs to water-soluble organic compounds which are required in minute quantities and are not synthesized by human/animal body. De novo biosynthesis of vitamin B_6 is limited to plants, bacteria, and fungi; therefore, man and animals rely on dietary sources (meat and fresh vegetables) to compensate vitamin B_6 requirement. Vitamin B_6 exists in six inter-convertable forms with similar biochemical properties. These forms are pyridoxamine (PM, an aminomethyl), pyridoxine (PN, a hydroxymethyl), pyridoxal (PL, an aldehyde), and their phosphorylated forms (Fig. 8.1), of which pyridoxal 5′-phosphate (PLP) is the most important form that plays a central role in many metabolic reactions as a cofactor (Hellmann and Mooney 2010; Ueland et al. 2017). Vitamin B_6 is also considered as an effective antioxidant and is associated with oxidative stress responses in both animals and plants (Bilski et al. 2000; Hellmann and Mooney 2010). Deficiency of vitamin B_6 results in many disorders such as hypertension, diabetes, heart disease, renal disorder, pellagra (Hellmann and Mooney 2010), and several neurological disorders like epilepsy, alzheimer, autism, schizophrenia, parkinson, etc. (Di Salvo et al. 2012). Vitamin B_6 deficiency is also a

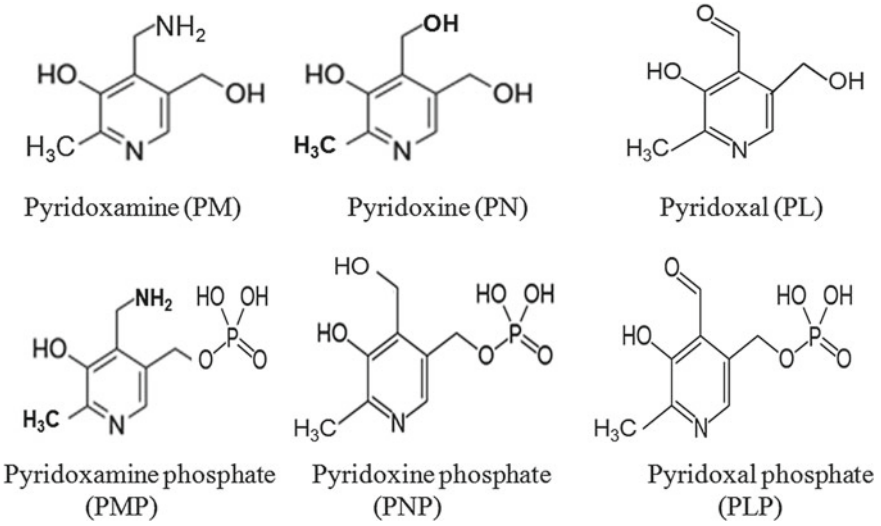

Fig. 8.1 Chemical structures of B_6 vitamers

risk factor for Nodding Syndrome in Northern Uganda (Obol et al. 2016) and Southern Sudan (Wadman 2011; Vogel 2012). Its deficiency threat can be enhanced by other factors like undernourishment, pregnancy, HIV, alcoholism, etc. (Fitzpatrick et al. 2012; Di Salvo et al. 2012). The key reason of vitamin B_6 deficiency in developing countries is the inadequate availability of food to poor people.

8.2.1 Food Sources of Vitamin B_6

Sunflower seeds, pistachios, fish, turkey, chicken, pork, beef, dried fruits, bananas, avocados, potatoes, cereals, and spinach, etc., are the foods which are rich in vitamin B_6 (Table 8.1). Potatoes contain the highest vitamin B_6 content compared to the other food crops (Fitzpatrick et al. 2012). Bioavailability of B_6 vitamers from vegetable source is associated with the sufficient intake of food (Vanderschuren et al. 2013). From the plant source, all the B_6 vitamers and their phosphorylated forms are completely bioavailable except PN-glucoside which is only 50% bioavailable (Gregory 2012). Physicochemical factors such as temperature, luminosity, and pH also affect B_6 vitamer content (Fitzpatrick et al. 2012). Pyridoxal (PL) and pyridoxal phosphate (PLP), the major types of vitamin B_6 found in animal tissues (Mehansho et al. 1979), are less stable than the pyridoxine (PN) and its glycosylated form (Vanderschuren et al. 2013).

Table 8.1 Some important food sources of vitamin B_6 (https://lpi.oregonstate.edu/mic/vitamins/vitamin-B6)

S. No.	Food	Vitamin B_6 (mg/100 g)
1	Fortified breakfast cereal	0.5–2.5
2	Salmon	0.48–0.80
3	Potato	0.70
4	Turkey	0.69
5	Avocado	0.52
6	Chicken	0.51
7	Spinach (cooked)	0.44
8	Banana	0.43
9	Dried plums, pitted	0.36
10	Hazelnuts (dry roasted)	0.18
11	Vegetable juice cocktail	0.13
12	Cassava	0.1
13	Wheat bran crude	1
14	Rice bran crude	4
15	Rice flour brown	1
16	Maize	0.4

8.2.2 Biosynthesis and Homeostasis of Vitamin B_6

In plants, vitamin B_6 is synthesized de novo in the cytoplasm. Two enzymes, pyridoxal phosphate synthase (PDX1) and glutaminase (PDX2), play key roles in synthesis of pyridoxal 5′-phosphate (PLP). This pathway is known as deoxyxylulose 5-phosphate (DXP)-independent pathway (Tambasco-Studart et al. 2005). On the other hand, DXP-dependent pathway involves seven enzymes and was first discovered in *Escherichia coli* (Fitzpatrick et al. 2007). Ammonia and glutamate are produced by the action of enzyme PDX2 from glutamine. The ammonia, glyceraldehydes 3-phosphate (G3P), and ribose 5-phosphate (R5P) are utilized as substrates by PDX1 for the synthesis of PLP (Fig. 8.2) (Colinas et al. 2016; Fudge et al. 2017; Strobbe and Van Der Straeten 2018).

A couple of enzymes like PMP/PNP oxidase (PDX3), PM/PN/PL kinase (SALT OVERLY SENSITIVE, SOS4), and non-specific phosphatases (PPase) are present all over in plastids, mitochondria, and the cytosol and operate during vitamin B_6 salvage pathway (Sang et al. 2007; Vanderschuren et al. 2013; Parra et al. 2018). The non-phosphorylated vitamers of vitamin B_6 (i.e., PM, PN, and PL) can be transformed to their phosphorylated forms by the action of SOS4 and non-specific phosphatases (PPase). Further, a pyridoxal reductase (PLR1) converts pyridoxal to pyridoxine through NADPH (Herrero et al. 2011; Strobbe and Van Der Straeten 2018) during cellular homeostasis of vitamin B_6 in plants (Fig. 8.3). The purine permease (PUP1) transports the pyridoxine and pyridoxal into the interior of cell from the guttation sap. Vitamin B_6 is necessary for regulation of plant growth and development, and

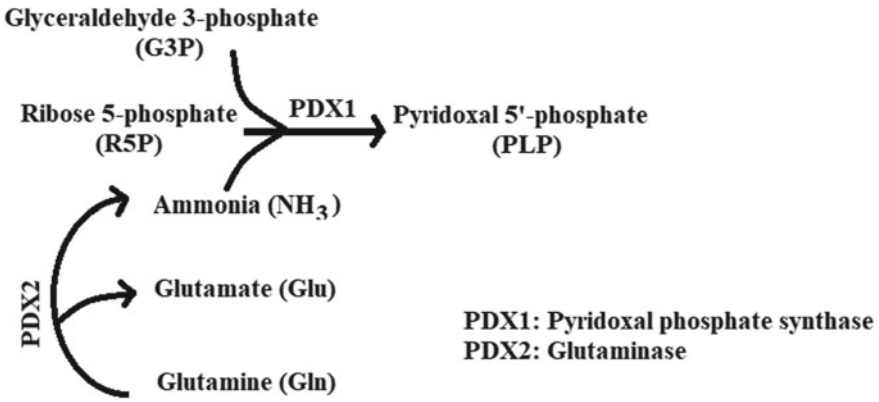

Fig. 8.2 De novo synthesis of PLP in plant cytosol

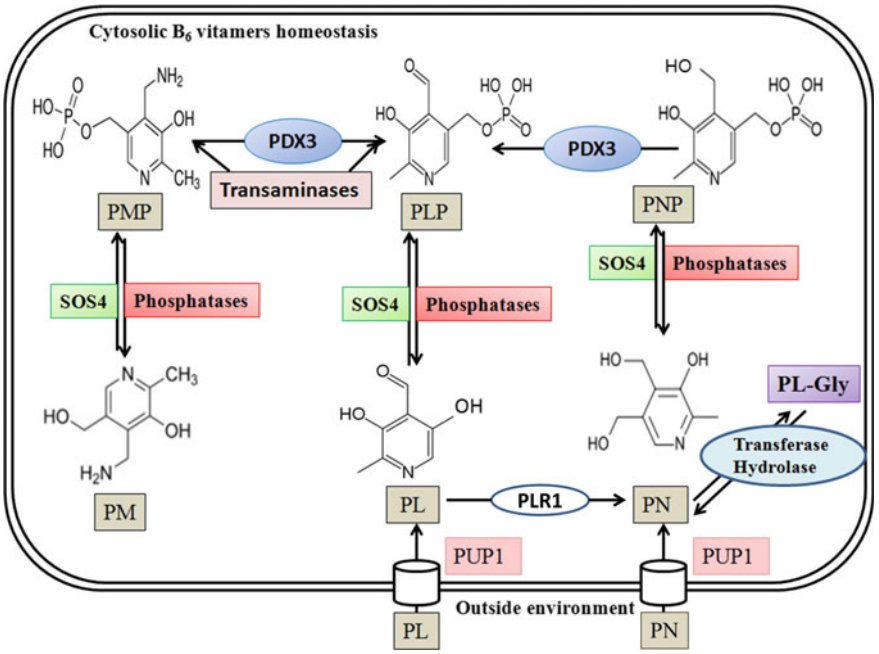

Fig. 8.3 Salvage pathway for synthesis of B_6 vitamers in plant cell decoded from *Arabidopsis thaliana* (modified after Fudge et al. 2017). Cytosolic B_6 vitamers homeostasis occurs through the action of a couple of enzymes. These are: the PNP/PMP oxidase, PDX3; the PL/PN/PM kinase, SOS4; the PL reductase, PLR1 non-specific phosphatases. Transaminases catalyze the cellular equilibrium of PMP/PLP. Transferases and hydrolases mediate the interconversion of PN and PL-Gly that are not yet characterized. PN and PL are taken from the external environment, i.e., guttation sap through the purine permeases, PUP1

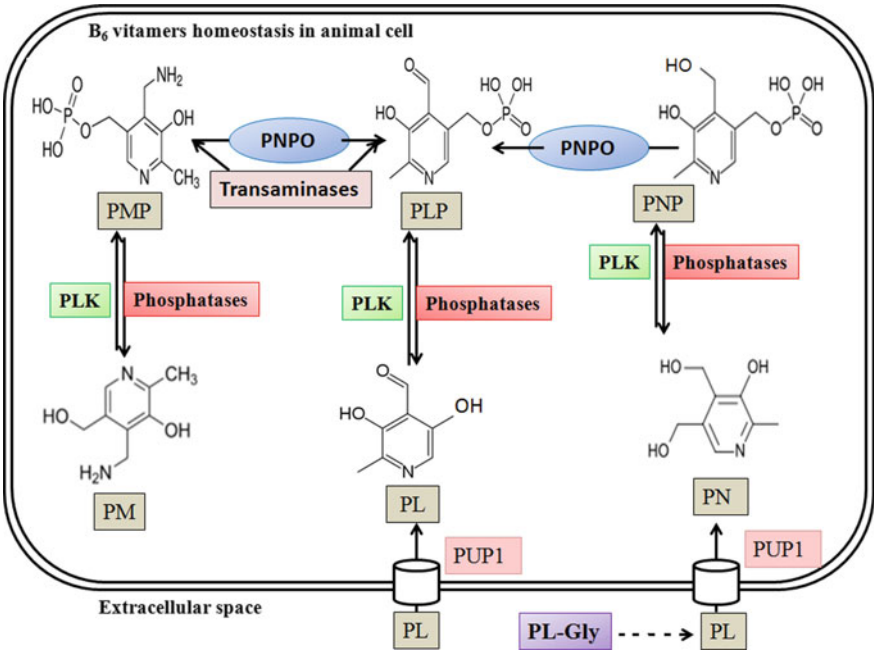

Fig. 8.4 B_6 vitamers homeostasis in animal cell (as decoded from *Homo sapiens*) (modified after Fudge et al. 2017). Which is comparable to that in plants and consists of the enzymes: PNP/PMP oxidase, PNPO; PL/PN/PM kinase, PLK; and phosphatases. Transaminases catalyze the cellular equilibrium of PMP/PLP. Unphosphorylated vitamers are taken up in the gut, i.e., extracellular space

the different forms of vitamin B_6 are balanced in the plants through the above-said process.

In animals/human, vitamin B6 homeostasis is similar to plants and includes the enzymes PNP oxidase (PNPO) in place of PDX3, PL/PN/PM kinase (PLK) in place of SOS4, non-specific phosphatase (PPase) and transaminase (Tases). Tases help in maintaining the cellular equilibrium of PMP/PLP. The non-phosphorylated vitamers can enter in the gut (i.e., extracellular space) (Fig. 8.4).

8.2.3 Vitamin B_6 Functions and Deficiency Diseases

Vitamin B_6, an important micronutrient required for proper human functioning, plays a central role as a cofactor/coenzyme for nearly 180 metabolic reactions, like transaminations, aldol cleavages, and carboxylations (Ueland et al. 2017). Further, vitamin B_6 also acts as an antioxidant (Justiniano et al. 2017) and helps in protein folding (Cellini et al. 2014) and the biosynthesis of heme and neurotransmitters (Ueland et al. 2017; Parra et al. 2018).

Deficiency of vitamin B_6 leads to a number of diseases in human like cardiovascular diseases, anemia, diabetes, rheumatoid arthritis, and various types of cancers such as lung, breast, kidney, and colorectal cancers (Ueland et al. 2017). Many neurological disorders like epileptic seizers (Skodda and Muller 2013) and peripheral neuritis (Ghavanini and Kimpinski 2014) are also related to the deficiency of vitamin B_6. Recent investigations on vitamin B_6 status at population level in developed and developing countries have shown that very low B_6 content is present in nearly one-in-four people in developed countries. The condition is more deteriorated in developing countries like Uganda and Sudan where about half of the population is vitamin B_6 deficient (Fudge et al. 2017). Humans cannot synthesize vitamin B_6 de novo and are completely dependent on dietary sources for vitamin B_6. However, bioavailability of vitamin B_6 is a major concern, as half of the B_6 pool (mainly from the plant food) is lost due to incomplete digestibility (Roth-Maier et al. 2002). Further, most of the food crops used worldwide like wheat, rice, potato, cassava, and maize are deficient in dietary vitamin B6 (Fudge et al. 2017) (Table 8.1).

8.2.4 Biofortification of Vitamin B_6

In the last decade, genetic engineering has emerged as a successful tool for enhancing the vitamin B_6 content in model plants like *Arabidopsis* (Chen and Xiong 2009; Raschke et al. 2011) and tobacco (Herrero and Daub 2007) and in staple crop plants like cassava (Li et al. 2015) and potato (Bagri et al. 2018) (Table 8.2). Vitamin B_6 pathway genes *PDX1* and *PDX2* are used for biofortification of vitamin B6. Crosstalk between biosynthesis and salvage pathways of vitamin B_6 occurs in plants which enable the B_6 vitamers to interconvert in cytosol according to the need of a plant (Tanaka et al. 2005). PDX1 and PDX2 are the two important cytosolic enzymes which are crucial for de novo biosynthesis of vitamin B_6. In higher plants, *PDX1* is present in multiple homologues, i.e., *PDX1.1*, *PDX1.2*, and *PDX1.3*, whereas *PDX2* is present as single homologue (Tambasco-Studart et al. 2005). The function of homologue *PDX1.2* has not been known till now. The similar homologues of both genes are identified in rice, cassava, and potato also (Ouyang et al. 2007; Prochnik et al. 2012; Mooney et al. 2013). The overexpression of *PDX1* and *PDX2* isolated from a pathogenic fungus, C*ercospora nicotianae*, in leaves of tobacco has increased the vitamin B_6 nearly by 20% (Herrero and Daub 2007). The overexpression of *PDX1.3* and *PDX2* genes in *Arabidopsis* under the control of a constitutive promoter 35S CaMV has increased vitamin B_6 by 1.2-fold, whereas when these genes are tagged with a seed-specific 12S promoter, the increase in vitamin B_6 was 1.4–3-fold. Further, the overexpression of *PDX1.1* alone and with *PDX2* has resulted in fourfold increase in shoots and seeds, and fivefold increase in seeds, respectively (Raschke et al. 2011). This study demonstrates that correct choice of homologues is important during genetic engineering. Interestingly, hyperaccumulation of vitamin B_6 has resulted in bigger seed size (through embryo enlargement) as well as larger

Table 8.2 Metabolic engineering of vitamin B_6 in plants

S. No.	Plant	Targeted genes	Increase in vitamin content	Plant part where increase occurs	Morphological changes in plants	Tolerant to stresses	Limitations	References
1	Tobacco haploid plants	*Cercospora nicotianae* genes, *PDX1* and *PDX2* under 35S CaMV	Modest increase (1.2-fold)	Leaf	Delayed seed germination and plant growth	No resistance to cercosporin or high salinity	Downregulation of endogenous genes	Herrero and Daub (2007)
2	*Arabidopsis*	Overexpression of *AtPDX1.3* and *AtPDX2* under 35S CaMV	1.2-fold	Seed	No effect on plant growth and development	No effect	NS	Chen and Xiong (2009)
		Overexpression of *AtPDX1.3* and *AtPDX2* seed-specific promoter 12S	Twofold	Seed	No change	NS	NS	
		Overexpression of *PDX1.1* alone	1.2-fold	Shoot and seed	Increase in seed[a] and aerial parts size	More resistance to high light and oxidative stress	Decrease in seed number and yield	Raschke et al. (2011)

(continued)

Table 8.2 (continued)

S. No.	Plant	Targeted genes	Increase in vitamin content	Plant part where increase occurs	Morphological changes in plants	Tolerant to stresses	Limitations	References
		Over-expression of *PDX1.1* and *PDX2*	Fourfold	Shoot and seed	Increase in seed[a] and aerial parts size		Decrease in seed number and yield	
3	Cassava	*AtPDX1.1* and *AtPDX2* with constitutive promoter 35S CaMV	14.8-fold	Uncooked leaves	No change detected	NS	NS	Li et al. (2015)
			9.0-fold	Cooked leaves				
		AtPDX1.1 and *AtPDX2* with tuber-specific promoter Patatin	5.8-fold	Uncooked roots	No change detected	NS	NS	
			Fourfold	Cooked roots				
4	Potato	*AtPDX2* constitutive promoter 35S CaMV	107–150%	Tuber	Increased tuber size	Improved tolerance to oxidative and salinity	NS	Bagri et al. (2018)
5	Rice	*AtPDX1.1* & *AtPDX2* with constitutive promoter 35S CaMV	28.3 fold 12-fold 3.1 fold	Leaves Roots Seeds (mainly in seed coat and embryo)	No effect on endogenous PDX expression and on plant growth & development	No enhanced tolerance to abiotic (salt) or biotic stress (resistance to Xanthomonas oryzae infection)	Affected seed yield in some lines	Mangel et al. (2019)

[a]Through embryo enlargement
NS Not specified

aerial organs, but the number of seeds harvested is decreased which results in low yield (Raschke et al. 2011). These transgenic lines are tolerant to various stresses.

Biofortification of vitamin B_6 by genetic engineering in cassava has shown promising results. The ectopic expression of *PDX1.1* and *PDX2* using constitutive promoter 35S CaMV and tuber-specific promoter (PAT) results in 14.8- and 5.8-fold increase in vitamin B_6 content of uncooked leaves and roots, respectively, with no change in morphology and physiology of plant (Li et al. 2015). The vitamin B_6 content in cooked transgenic cassava leaves and roots was nine and fourfold higher compared to the wild types. Moreover, these transgenic plants were stable in field conditions and vitamin B_6 contents were bioavailable (Li et al. 2015). Recently, the overexpression of a single gene *PDX2* under the control of constitutive promoter 35S CaMV in potato has increased vitamin B_6 content by 107–150% (Bagri et al. 2018). The tuber size has also increased due to the accumulation of PDX2 proteins and plants have shown tolerance to various stresses (Bagri et al. 2018; Table 8.2). The results obtained from the studies on cassava and potato are encouraging and conclude the success of two gene engineering strategies (Li et al. 2015) but open the options for engineering one gene (Bagri et al. 2018). More studies are required to establish these strategies for biofortification in other economically important crop plants. Further, till now there is no report of biofortification of monocot plants especially wheat and maize which are used as staple food crops. However, rice transgenic plants constitutively expressing Arabidopsis *PDX1.1* and *PDX2* genes have shown increase in vitamin B_6 content in leaves (up to 28.3 fold), roots (12 fold) and seeds (3.1 fold mainly in seed coat and embryo with little in endosperm) with no effect on overall growth and tolerance to abiotic (salt stress) or biotic stress (resistance to *Xanthomonas oryzae* infection) (Mangel et al. 2019).

8.3 Ascorbic Acid

Vitamin C or L-ascorbic acid (L-threo-hex-2-enono-1,4 lactone, ascorbate) is a vital water-soluble micronutrient found in eukaryotes. Ascorbic acid in water solutions forms a monovalent anion, ascorbate, which donates electrons to oxidized molecules. It is a key antioxidant that protects the cells and cell organelles from harmful effects of reactive oxygen species (ROS). It helps the human body in fighting against several oxidative stress-related diseases like cardiovascular, cancer, aging, etc., and boosts the immunity. Most of the animals synthesize ascorbate as they have L-gulono-1,4-lactone oxidase (GULO) that catalyzes the last step in ascorbic acid synthesis, but humans along with other primates do not synthesize their own ascorbate due to the mutations in GULO gene, and thus, they obtain it from their diet. Being an essential human micronutrient, its deficiency causes scurvy, a disease which is rare now but was discovered by the sailors as early as 1497 due to the non-consumption of fresh plant-derived food for months. The symptoms of scurvy includes joint pain, swollen and bleeding gums, skin lesions due to ruptured blood vessels, and in severe cases can result in death. In 1747, it was demonstrated that the consumption of

citrus fruits which are rich in vitamin C can cure/prevent scurvy. Hence, vitamin C was called as 'antiscorbutic factor.' Albert Szent-Gyorgyi isolated the antiscurvy molecule 'vitamin C' in 1928. It was crystallized in 1932, and its structure was elucidated in 1933. Humans meet their daily requirement of ascorbic acid from the plant-derived food. The recommended daily allowance (RDA) of vitamin C varies with countries, e.g., 40 mg/day for adult man in UK, 70 mg/day in Netherlands, 90 mg/day in USA, and 100 mg/day in Germany (Troesch et al. 2012). A daily dose of 90–100 mg vitamin C has been recommended to prevent heart diseases, cancer, and cataract, in contrast to 45 mg/day required to protect from scurvy (Carr and Frei 1999). Increasing its RDA to 200 mg/day would provide enhanced health benefits (Frei et al. 2012). Humans need 10 mg/day although the RDA for ascorbate is 1 mg/kg/day. Vitamin C deficiency affects 10–14% US adults (Velandia et al. 2008). In developing countries, people are largely dependent on cereals for energy requirement which are deficient in vitamin C. Plants with higher ascorbate levels are to be developed for not only to provide the health benefits but also to improve their performance under increased earth temperature and water scarcity due to the recent rise in CO_2 and other greenhouse gases. Developing stress-tolerant crops ensure food and nutritional security for overgrowing population. Producing plants with higher ascorbate levels is an imperative approach to develop climate-resilient crops.

8.3.1 Ascorbic Acid Market

L-ascorbic acid (AsA) has the largest share in the global vitamins market. It is used in several industries, e.g., pharmaceutical, food, beverages, cosmetics, and feed (Camrena and Wang 2016; Moser and Chun 2016). The pharmaceutical industry is the largest consumer of ascorbic acid. The global market for ascorbic acid and its derivatives is estimated to >110,000 tons per year and $1 billion (Austria et al. 1997; Pappenberger and Hohmann 2014). Eighty percent of the world's supply for ascorbic acid comes from China. The industrial production of ascorbic acid is not efficient and costly. However, plant-derived food has higher bioavailability of ascorbic acid than the synthetic/purified molecule used in supplementation (Vissers et al. 2011). This may be due to the presence of several antioxidants/redox molecules in plant food that are synergistic to ascorbic acid or maintain ascorbic acid in its active reduced state (Villanueva and Kross 2012).

8.3.2 Ascorbate Biosynthesis

Ascorbate is synthesized only by eukaryotes and not by the prokaryotes. Several eukaryotes like primates (including man), bats, guinea pig, and teleost fish do not have functional *GULO* gene that encodes the last enzyme of ascorbate biosynthetic pathway thus they acquire it from their diet. AsA biosynthesis pathways vary between

plants, animals, and photosynthetic protists (Wheeler et al. 2015). Fungi produce a 5C analogue, D-erythroascorbate. Ascorbate biosynthesis pathways have been reviewed previously (Loewus 1999; Smirnoff and Wheeler 2000; Valpuesta and Botella 2004; Wheeler et al. 2015; Bulley and Laing 2016), but an overview is presented here (Fig. 8.5). In higher plants, ascorbic acid is mainly synthesized from D-glucose without inversion of carbon chain through the Smirnoff–Wheeler pathway (Wheeler et al. 1998). D-glucose is converted by hexokinase to glucose-6-phosphate which is further converted to GDP-D-mannose through four reversible steps catalyzed by PGI, PMI, PMM, and GMP/VTC1. GDP-D-mannose is converted into L-galactose by three steps catalyzed by GME, GGP/VTC2, and GPP/VTC4. L-galactose is reduced by galactose dehydrogenase (GDH) into L-galactono-1,4-lactone, and galactono-1,4-lactone dehydrogenase reduces it further to ascorbate.

The **second** pathway of ascorbate synthesis involves the reduction of the substrate D-galacturonate (D Gal) (derived from pectin degradation by enzymes, pectin methylesterases, PMEs and polygalacturonases, PGs) to L-galactonate (by the

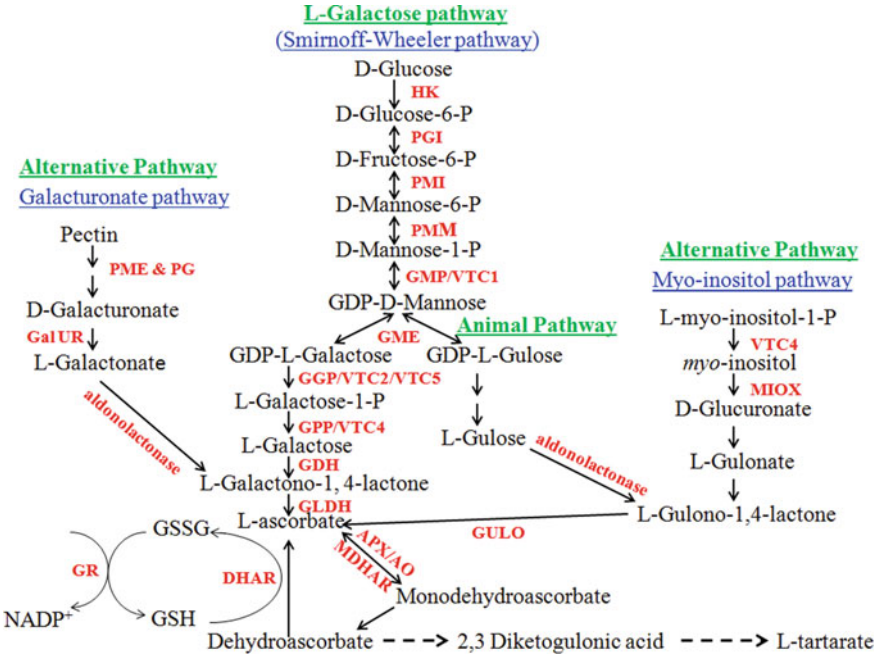

Fig. 8.5 Major biosynthetic and recycling pathways of ascorbate in plants. Enzymes are highlighted in red color. HK: hexokinase; PGI: phosphoglucose isomerase; PMI: phosphomannose isomerase; PMM: phosphomannomutase; GMP: GDP-D-mannose pyrophosphorylase (VCT1); GME: GDP-D-mannose-3′,5′-isomerase; GGP: GDP-L-galactose phosphorylase (VTC2/VTC5); GPP: L-galactose-1-phosphate phosphatase (VCT4); GDH: L-galactose dehydrogenase; GLDH: L-galactose-1,4-lactone dehydrogenase; APX: ascorbate peroxidase, MDHAR: monodehydroascorbate reductase; DHAR: dehydroascorbate reductase; GR: glutathione reductase; MIOX: *myo*-inositol oxygenase; GULO: L-gulonolactone oxidase; GalUR: D-galacturonate reductase

enzyme L-galactose dehydrogenase, GalUR) which is spontaneously converted to L-galactono-1,4 lactone. This compound feeds into the terminal step of L-galactose pathway where it is reduced to ascorbate via the action of L-galactono-1,4-lactone dehydrogenase (GLDH) enzyme.

The **third** pathway is similar to the main pathway that starts from D-glucose, but branch off from GDP-D-mannose which is converted to L-gulose in three reactions catalyzed by GDP-D-mannose epimerase (GME), GDP-L-gulose-1-P phosphatase, and L-gulose-1-P phosphatase, respectively. The L-gulose is reduced to L-gulono-1,4 lactone which then oxidizes to ascorbate by L-gulonolactone oxidase (GULO).

The **fourth pathway** involves the synthesis of ascorbate from myo-inositol. In short, myo-inositol is converted to L-gulono-1,4-lactone in three steps catalyzed by myo-inositol oxygenase (MIOX), glucuronate reductase, and aldonolactonase (Valpuesta and Botella 2004). L-gulono1,4-lactone is finally converted in ascorbate by the action of L-gulonolactase.

Thus, plants have several routes but the contribution of each of these pathways varies between different species, organs, and developmental stages.

8.3.3 Ascorbate Functions

The functions of ascorbate are well-reviewed elsewhere (see Smirnoff and Wheeler 2000; Smirnoff 2018), and an overview is presented here. Ascorbate is an antioxidant for detoxification of free radicals generated during metabolism or under stress conditions and acts as a cofactor for many enzymes like violaxanthin de-epoxidase (uses ascorbate as reductant to protect photosynthetic damage by intense light), myrosinase (a thioglucosidase that uses ascorbate for the synthesis of glucosinolates), and dioxygenases (Fe^{2+}/2-oxoglutarate-dependent dioxygenases, 2-ODDs with iron on its active site, uses ascorbate as reductant). Ascorbate reduces ferric (Fe^{3+}) to ferrous (Fe^{2+}) for iron uptake and transport (to overcome iron deficiency, anemia), or requires the dioxygenase enzymes like prolyl hydroxylase (to hydroxylate the proline residues of collagen) or DNA and histone demethylases (that participate in regulation of epigenetic mechanisms controlling cell differentiation, whose dysregulation can result in certain types of cancers). Ascorbate prevents inactivation 2-oxoglutarate-dependent dioxygenase by reducing active center Fe^{2+}. Ascorbate is also involved in plant development, cell cycle, cell expansion flowering, hormone signaling, fruit ripening, and senescence.

8.3.4 Ascorbic Acid Content

The ascorbate content of plants varies with cultivars of a species, between species and different tissues of the same plant (Gest et al. 2013). Ascorbate levels in the plums of the kakadu (*Terminalia ferdinandiana*) are as high as 5300 mg/100 g FW followed

by in cherries of Acerola (*Malpighia emarginata*) with 2800 mg/100 g FW, whereas in fruits of wild accessions of *Actinidia chinensis* var *chinensis* and *Hippophae rhamnoids* (sea buckthorn) contain 400–420 mg/100 g FW. Most of the main staple food crops except potato and cassava have less than 10 mg ascorbate/100 g FW which further declines upon their storage or processing and cooking. Increase in ascorbate content in these crops would deliver additional health benefits to a large section of the society and improve their tolerance to a wide range of stresses. Variations in ascorbate content in edible tissues of different cultivars have been reported in potato (Augustin et al. 1978). Such intra-species variations can explain differences among cultivars for abiotic and biotic stress tolerance.

8.3.5 Strategies for Ascorbate Biofortification

8.3.5.1 Biotechnological/Metabolic Engineering Approaches

Strategies based on the overexpression of the genes involved in ascorbate metabolism (biosynthesis or recycling or regulation) or inhibition of degradation/catabolism have used with varying degree of success in achieving elevated ascorbate levels in plants (Locato et al. 2014; Macknight et al. 2017; Mellidou and Kanellis 2017; Strobbe et al. 2018; Fenech et al. 2019) (Table 8.3). Increase in ascorbate content is beneficial for plant stress tolerance and improves their nutritional value, to prevent losses due to long-term storage/post-harvest degradation or browning.

8.3.5.2 Improvement in Ascorbate Levels by Manipulating Main Biosynthetic Pathway

AsA in higher plants is mainly synthesized from D-glucose through Man/L-galactose pathway (Smirnoff and Wheeler pathway) (Fig. 8.5). The intermediate steps, enzymes, and genes of this pathway have been identified using the fully characterized vitamin C deficient *Arabidopsis* mutants (*vtc*) and other molecular biological tools (Conklin et al. 2006; Laing et al. 2007; Maruta et al. 2008). The S-W pathway's intermediate, GDP-D-mannose (and to a much less extent GDP-L-galactose), is also used for the synthesis of cell wall polysaccharides and glycoproteins. The enzymes upstream of this step such as phosphomannose isomerase (PMI) and phosphomannomutase (PMM) do not have major influence over AsA homeostasis (Qian et al. 2007; Maruta et al. 2008). The role of GDP-D-mannose pyrophosphorylase (GMP or VTC1) and GDP-D-mannose-3,5 epimerase (GME) enzymes of the L-galactose pathway is extremely debatable. GMP expression is correlated with AsA concentration in acerola (Badejo et al. 2009) but not in kiwifruit (Bulley et al. 2009), tomato (Ioannidi et al. 2009), or blueberry (Liu et al. 2015). Good association between GME transcripts and AsA is reported in apple (Li et al. 2010a) and blueberry (Liu et al. 2015) but not in tomato (Ioannidi et al. 2009; Mellidou et al. 2012) or kiwifruit

Table 8.3 Engineering ascorbate in plants for biofortification or stress tolerance[a]

Pathway	Transgenic plant	Transgene	Transgene source	Tissue examined	Max fold increase in AsA	Stress tolerance	Growth	References
L-galactose	Tomato	*GGP*	Kiwi	Fruit	6.2	–	↓Yield	Bulley et al. (2012)
	Potato	*GGP*	Potato	Tuber	3	–	–	
	Strawberry	*GGP*	Kiwi	Fruit	2.1	–	–	
	Arabidopsis	*GGP*	*Arabidopsis*	Leaves	2.9	–	–	Zhou et al. (2012)
	Arabidopsis	*GGP* + *GPP*	*Arabidopsis*	Leaves	4.0	–	–	
	Arabidopsis	*GGP* + *GLDH*		Leaves	3.5	–	–	
	Arabidopsis	*GPP*		Leaves	1.6	–	–	
	Arabidopsis	*GLDH*		Leaves	1.4	–	–	
	Arabidopsis	*GMP*		Leaves	1.4	–	–	
	Arabidopsis	*GDH*		Leaves	1.3	–	–	
	Arabidopsis	*GME*		Leaves	1.3	–	–	Zhou et al. (2012)
					1.6	Drought, salt and acid	–	Ma et al. (2014)
	Rice	*GGP* and *GME*	*Arabidopsis*	Leaves	2.5 and 1.4	Salt	–	Zhang et al. (2015a, b)
	Tobacco	*GGP* and *GME*		Young and old leaves	1.0	–	–	Imai et al. (2012)

(continued)

Table 8.3 (continued)

Pathway	Transgenic plant	Transgene	Transgene source	Tissue examined	Max fold increase in AsA	Stress tolerance	Growth	References
	Tomato	*GMP*	Yeast	Fruit (red)	1.3	–	–	Cronje et al. (2012)
	Tobacco	*GLDH*	*Rosa roxburghii*	Leaves	2.1	Salt and herbicide	–	Liu et al. (2013)
	Lettuce	*GLDH*		Leaves	1.3	–	–	Landi et al. (2015)
Alternative pathway								
Myo-inositol	Lettuce and tobacco	GLOase	Rat	–	2–7	–	–	Jain and Nessler (2000)
	Arabidopsis	GLOase	Rat	–	2–3		↑	Radzio et al. (2003)
	Arabidopsis	*MIOX4*	*Arabidopsis*	–	1.5–2	Salt and temperature	↑	Lisko et al. (2013)
	Arabidopsis	*MIOX4*	*Arabidopsis*	–	2–3	–	–	Lorence et al. (2004)
	Arabidopsis	*MIOX4*	*Arabidopsis*	–	1.7	Temperature	↑NPQ	Tóth et al. (2011)
	Arabidopsis	*MIOX4*	*Arabidopsis*	–	1			Endres and Tenhaken (2009)

(continued)

Table 8.3 (continued)

Pathway	Transgenic plant	Transgene	Transgene source	Tissue examined	Max fold increase in AsA	Stress tolerance	Growth	References
	Tomato	*MIOX4*	*Arabidopsis*	–	2	–	–	Kulkarni (2012)
	Tomato	*MIOX2*	*Arabidopsis*		1			Cronje et al. (2012)
	Arabidopsis	*AtPAP15*	*Arabidopsis*	–	2	–	–	Zhang et al. (2008)
D-Galacturonate	*Arabidopsis*	*GalUR*	Strawberry	–	2–3	–	–	Agius et al. (2003)
	Tomato (hairy roots)	*GalUR*	Strawberry	–	2		↓	Wevar-Oller et al. (2009)
	Tomato	*GalUR*	Strawberry	–	2.5	Oxidative, salt, drought	–	Lim et al. (2016)
	Tomato	*GalUR*	Strawberry	–	1.5–2.5	Oxidative, salt, light	–	Cai et al. (2015)
	Potato	*GalUR*	Strawberry	–	2	Salt, drought	–	Hemavathi et al. (2009), Upadhyaya et al. (2009)
Recycling pathway	Tobacco and maize	*DHAR*	*Arabidopsis*	Leaves	2	Ozone, salt, drought	–	Eltyeb et al. (2006)
	Arabidopsis	*DHAR*	Rice	Leaves	1–1.5	Oxidative, salt, temperature	–	Ushimaru et al. (2006)

(continued)

Table 8.3 (continued)

Pathway	Transgenic plant	Transgene	Transgene source	Tissue examined	Max fold increase in AsA	Stress tolerance	Growth	References
	Tobacco	*DHAR*	Tobacco	–	3	Ozone	↑Assimilation	Chen and Gallie (2005)
	Potato	*DHAR*	Sesame	Leaves	1.5	–	–	Goo et al. (2008)
	Potato	*DHAR*	Potato	Leaves	1–1.8	–	–	Qin et al. (2011)
	Maize	*DHAR*	Rice	–	6			Naqvi et al. (2009)
	Arabidopsis	*DHAR*	*Arabidopsis*	Leaves	2–4	Oxidative, temperature, light	–	Wang et al. (2010)
	Tomato	*DHAR*	Tomato	Fruit	1.6	–	–	Haroldsen et al. (2011)
	Tomato	*DHAR*	Tomato	–	1–1.5	Oxidative, salt	–	Li et al. (2012a, b)
	Tomato	*DHAR*	*Pyrus sinkiangensis*	–	1.5	Salt, temperature	–	Qin et al. (2015)
	Tomato	*DHAR*	Tomato	–	1.27	Oxidative salt, temperature	–	Gest et al. (2013)
	Arabidopsis	*DHAR*	Kiwi	–	1–1.2	–	–	Liu et al. (2015)

(continued)

Table 8.3 (continued)

Pathway	Transgenic plant	Transgene	Transgene source	Tissue examined	Max fold increase in AsA	Stress tolerance	Growth	References
	Arabidopsis	*DHAR*	*Arabidopsis*	–	1–1.2	Oxidative	–	Yin et al. (2010)
	Arabidopsis	Chl-*MDHAR*	Tomato	–	1–1.2	Oxidative, light	↑A↑Fm/Fv	Li et al. (2010b)
Alternative/Regulatory factors	Tomato	*MDH*	Tomato	–	6	–	↑A↑rETR	Nunes-Nesi et al. (2005)
	Tomato	*ALO*	Yeast	–	1.5	–	–	Cronje et al. (2012)
	Tobacco	*ALO*	Yeast	–	2–3	Drought, temperature	–	Bao et al. (2016)
	Tobacco	*ALO*	Yeast	–	2	Oxidative, light	–	Chen et al. (2015)
	Stylosanthes	*ALO* + *SgNCED*	Yeast + *Stylosanthes*	–	3–4	Temperature	↑Fm/Fv	Bao et al. (2016)
	Tomato	*SlHZ24*	Tomato	–	1.5	Oxidative	–	Hu et al. (2016)
	Arabidopsis	*AMR1* mutant	*Arabidopsis*	–	2–3	Ozone	–	Zhang et al. (2009)
	Arabidopsis	*Aterf98*	*Arabidopsis*	–	1.7	Salt	–	Zhang et al. (2012)
	Arabidopsis	*KJC1*	*Arabidopsis*	–	1.5	–	–	Sawake et al. (2015)

(continued)

Table 8.3 (continued)

Pathway	Transgenic plant	Transgene	Transgene source	Tissue examined	Max fold increase in AsA	Stress tolerance	Growth	References
	Tomato	*SIDOF22*	RNAi silencing	–	1.2	Salt	–	Cai et al. (2016)
	Arabidopsis	*CML10* mutant	*Arabidopsis*	Seedlings	−1.4	–	–	Cai et al. (2016)
	Arabidopsis	*VTC3* mutant	*Arabidopsis*	Leaves	−3.8	Temperature, light stress	–	Conklin et al. (2013)
	Arabidopsis	*CSN5B* mutant	*Arabidopsis*	Seedlings	1.4	–	–	Wang et al. (2013)
	Tobacco and *Pyrus ussuriensis*	WRKY transcription factor (*PbrWRKY53*)	*Pyrus betulaefolia*	Plant	More AsA accumulation via activation of *PbrNCED1* expression	Drought tolerant	–	Liu et al. (2019)
Gene editing via CRISPR/Cas	Lettuce	uORF mutant of lettuce homologue of *At VTC2* (GDP-L-galactose phosphorylase (LsGGP2))	Lettuce	Leaves	150%	Oxidative stress	–	Zhang et al. (2018)
	Tomato	uORF mutant of GGP	Tomato	Leaves	1.5 fold	–	–	Li et al. (2018b)

[a]Modified after Macknight et al. (2017) and George et al. (2017)

(Bulley et al. 2009). Tomato plants expressing a yeast *GMP* gene under a constitutive promoter has increased the AsA in the leaves by 70% and in green fruits by 50%, but in red fruits by only 35% (Cronje et al. 2012). Comparable increases were observed in tomato plant overexpressing *GMP3* (Zhang et al. 2013). Modification of *GME* expression is found to have very little effect on AsA levels either in leaf or fruit tissues (Bulley et al. 2009; Zhang et al. 2010; Mounet-Gilbert et al. 2016). For example, *GME* overexpression in the leaves of tomato and rice results in moderate increase in AsA levels (Zhang et al. 2012, 2015a, b). The GDP-L-galactose phosphorylase (GGP/VTC2) catalyzing the first committed step in ascorbate biosynthesis is rate limiting and thus a major target for metabolic engineering (Bulley and Laing 2016). Overexpression of kiwi or *Arabidopsis GGP* gene in strawberry, potato, and tomato under a constitutive promoter (35S) results in very significant fruit/tuber ascorbate levels (Bulley et al. 2012) indicating the overexpression of *GGP* consistently and significantly increased ascorbate contents in different species. The tomato plant overexpressing kiwi *GGP* gene has shown six-fold ascorbate in fruits (with developmental defects) and no seed setting but normal strawberry fruits. Besides the well-documented transcriptional control of GGP in regulation of ascorbate amount, its translation is also feedback controlled by ascorbate (Laing et al. 2015). This regulation occurs through conserved cis-acting upstream open reading frame (uORF, with conserved ACG codon) present at 5′ untranslated region of GGP that represses the translation of the downstream GGP ORF under high ascorbate concentrations. Disruption of this ORF through gene editing (CRISPR/Cas) in lettuce has resulted in increase of ascorbate content by 150% as well as tolerance to oxidative stress (Zhang et al. 2018). Similar disruption of tomato *GGP* by CRISPR/Cas has also increased ascorbic acid by 1.5-fold in leaves (Li et al. 2018b).

The next gene of the pathway, L-galactose-1-P phosphatase (GPP or VTC4), is not found to be rate limiting in AsA biosynthesis (Li et al. 2017). None of the genes downstream, L-galactose dehydrogenase (*GalDH*) and *GLDH* in tomato exert a considerable effect on ascorbate pool but *GLDH* regulates ascorbate content in pepper probably by its involvement in the transport of ascorbate among different organs (Rodríguez-Ruiz et al. 2017).

8.3.5.3 Improvement of Alternative Ascorbate Biosynthetic Pathways

Ascorbate biosynthesis through the precursor, D-galacturonate (called D-galacturonate pathway) which is derived from pectin degradation by pectin esterase and polygalacturonase, is reported in different species such as strawberry (Agius et al. 2003), grape (Cruz-Rus et al. 2010), apple (Mellidou et al. 2012), orange (Xu et al. 2012), and rose (Li et al. 2017) or at a specific developmental stage, e.g., ripe tomato fruit (Badejo et al. 2012). Overexpression of pectin esterase or polygalacturonase to increase pectin degradation is not a realistic strategy to increase ascorbate content, since pectin degradation might decrease shelf life of tomato fruit (Locato et al. 2014). However, PME activity has a modest role in tomato fruit softening (Brummel and Harpster 2001). Tobacco plants overexpressing a specific PME inhibitor

(*PMEI*) have shown reduction in PME and decreased ascorbate content (Lionetti et al. 2015). *Solanum pennellii* introgression line (IL12-4-SL) contains one QTL locus that increases ascorbate content in tomato fruit (Ruggieri et al. 2015) that might be due to pectin de-methylesterification/degradation (Rigano et al. 2018). Overexpression of tomato *GalUR* in *Arabidopsis* and strawberry *GalUR* in potato tubers and tomato fruits significantly increased ascorbate concentration (Agius et al. 2003; Hemavathi et al. 2009; Amaya et al. 2015). Tomato lines overexpressing strawberry *GalUR* have shown enhanced oxidative stress resistance, cold and salt tolerance, and displayed higher fruit number and yield (Cai et al. 2015; Lim et al. 2016).

The other alternative ascorbate synthesis pathways are: a side branch of L-galactose pathway that operates through GDP-gulose, a product of one of the two epimerization reactions catalyzed by GDP-D-mannose epimerase (GME) (Wolucka and Van Montagu 2003) or through myo-inositol pathway where *myo*-inositol generates D-glucuronate. GDP-gulose and D-glucuronate are finally converted into gulono-1,4-lactose (GulL) which is then transformed into ascorbate through the action of the terminal enzyme, gulonolactone oxidase (GULO). The overexpression or knockout of *MIOX* does not significantly contribute to ascorbate production (Endres and Tenhaken 2009). However, overexpression of rat *GULO* gene in several plant species has significantly increased ascorbate levels (Jain and Nessler 2000; Lim et al. 2012). Ectopic expression of this gene in all the five *Arabidopsis* vitamin C mutants (*vtc*) restored leaf ascorbic acid content indicating that the introduction of a novel pathway of ascorbic synthesis but not that the pathway is active in wild plants (Radzio et al. 2003). Further work on knockout of these enzymes is required in order to assess the contribution of these pathways in ascorbate synthesis in plants.

8.3.5.4 Improvement in Ascorbate Recycling

The plant's AsA pool is not only generated through de novo synthesis but also through the AsA recycling pathway. AsA oxidation product, the short-lived monodehydroascorbate (MDHA), regenerates AsA by MDHA reductase (MDHAR) or disproportionate spontaneously into AsA and dehydroascorbate (DHA). DHA is recycled to AsA by DHA reductase (DHAR) which uses glutathione (GSH) as the reductant through the cycle called ascorbate–glutathione cycle. If DHA is not reduced back to AsA, an irreversible loss of ascorbate occurs through its degradation into oxalate and threonate (Truffault et al. 2017). Efficient recycling of AsA will recover protection of the ascorbate pool from degradation and thus improve ascorbate content of plants. DHAR transcript levels have resulted in increased AsA accumulation in blueberry (Liu et al. 2015), tobacco (Eltayeb et al. 2006), rice leaves (Kim et al. 2013), *Arabidopsis* leaves (Wang et al. 2010), maize leaves and kernels (Chen et al. 2003; Naqvi et al. 2009), and tomato fruits (Qin et al. 2015). In potato, overexpression has improved the total pool of AsA but not to the extent as in other plants (Qin et al. 2011). In tomato, such increase has improved tolerance against oxidative, salt and temperature stresses (Qin et al. 2015).

The QTL mapping and introgression lines (Sauvage et al. 2014), expression, and activity profiles of MDHAR during tomato ripening (Mellidou et al. 2012) have demonstrated its main role in controlling AsA pool. Overexpression of *MDHAR* has been found to have either no effect on AsA content in tobacco and tomato (Yin et al. 2010; Haroldsen et al. 2011) or negative effect on AsA content (Gest et al. 2013). Tomato lines underexpressing *MDHAR* have shown a slight decrease in ascorbate degradation products indicating improvement in protection of AsA pool (Truffault et al. 2017). Additionally, reduction in *MDHAR* through RNAi approach has decreased tolerance to cold storage and AsA levels in tomatoes (El Airaj et al. 2013). Overexpression of chloroplastic or peroxisomal isoform of *MDHAR* in tomato (Li et al. 2010b) and tobacco (Eltayeb et al. 2006), respectively, has improved AsA concentration, indicating the different role of the organelle-specific isoform on AsA pool. Thus, improvement in regeneration efficiency of recycling enzymes may be carried further because a number of QTLs has been found to be associated with DHAR and MDHAR.

8.3.5.5 Improvement in Regulation of Ascorbate Biosynthesis

Several genes responsible for regulation of the ascorbate levels have been identified and characterized (Table 8.3). Some of them have positive effect on the AsA levels and are classified as transcription factors (TF), e.g., ETHYLENE RESPONSE FACTOR 98 (ERF98) (Zhang et al. 2012) or HD-ZIP (Hu et al. 2016). ERF98 is induced by ethylene, salt, and H_2O_2 and transcriptionally activates GMP (VCT1) to improve ascorbate synthesis in *Arabidopsis*. A tomato HD-Zip 1 transcription factor, SIHZ24, that binds to the promoter of an ascorbate biosynthetic gene encoding GDP-D-mannose pyrophosphorylase 3 (*SIGMP3*) modulates *SIGMP3* transcription and increases ascorbate levels. Overexpression and downregulation of SIHZ24 have been shown to increase and decrease SIGMP3 expression. SIHZ24 transcription factor also targets several other genes of ascorbate biosynthesis like *SIGME 2* and *SIGGP* by binding to their promoters. SIHZ24 overexpression lines promote ascorbate biosynthesis and enhance oxidative stress tolerance (Hu et al. 2016). A novel regulatory gene, *SIZF3* (encodes for C2H2-type zinc finger protein from tomato), competes with VTC1 to bind to CSN5B (a component of COP9 signalosome), and this competition inhibits the degradation of VTC1 through 26S proteosome. *SIZF3* overexpression in tomato and *Arabidopsis* promotes accumulation of ascorbate and enhances their salt tolerance by scavenging ROS (Li et al. 2018a).

Some of the factors, CSN5B and F-box protein AMR1, repress ascorbate synthesis, as a decrease in their expression increases ascorbate concentration or their overexpression decreases ascorbate. A decrease in *AMR1* expression led to twice increase in ascorbate concentration and transcript levels of GGP, GME, and GMP (Zhang et al. 2009). CSN5B has been found to interact with GMP and is involved in light–dark control of ascorbate biosynthesis. CSN5B promotes GMP (VTC1) degradation through the 26S proteosome in the dark to decrease ascorbate content

(Wang et al. 2013). A point mutation in *VTC1* increased ascorbate biosynthesis and *Arabidopsis* seedling growth (Li et al. 2016).

Some factors affect the enzymes or TF concentrations, such as VTC 3 (Conklin et al. 2013), CSN5B, a subunit of constitutive photomorphogenesis (COP9) signalosome (Wang et al. 2013), and ascorbate mannose pathway regulator-1 (AMR1) (Zhang et al. 2009). VTC3 is constitutively expressed over a wide range of conditions to influence the ascorbate pool at post-transcriptional level (Conklin et al. 2013). It is also suggested that VTC3 is also involved in the uORF regulation of GGP (Bulley and Laing 2016). The factors that directly affect enzyme activity are two nucleotide sugar pyrophosphorylase-like proteins, KONJAC 1 (KJC1) and KCJ2 (Sawake et al. 2015) and a calmodulin like (CML) protein, CML10 (Cho et al. 2016). KONJAC 1 and 2 stimulate the activity of GMP (VTC1). *KJC* mutants lack KJC proteins and showed reduction in GMP activity and significantly lowered AsA level. *KJC 1* overexpression significantly increased GMP activity. CML10 promotes accumulation of ascorbate by direct interaction with phosphomannomutase (PMM). *cml 10* knockdown mutants (amiRNA lines) and *pmm-12* mutants (point mutation lines) cannot produce sufficient ascorbate to scavenge excessive amount of ROS (Cho et al. 2016). Most of the studies have been carried out on model plants, and it is yet to be seen whether these factors functions similarly in crop plants. Further research is required to find the factors involved in regulation of ascorbate biosynthesis and metabolism for enhancing human nutrition by biofortifying crops and improving tolerance to abiotic stresses.

8.3.6 Transport/Sub-cellular Localization of Compartment Enzymes

In plants, ascorbate is present in all tissues and cell compartments as well as in the extracellular space (apoplast), but the distribution and concentration vary between tissues and are affected under different environment conditions and developmental stages. Ascorbate concentration is maximum in leaves and flowers and lesser in stems and roots. It is pertinent to consider that the effective translocation and accumulation of AsA at all the sites of the cell may be due to simple diffusion. Since AsA is negatively charged at physiological pH values and therefore is unable to diffuse through lipid bilayer. The involvement of a large family of transporters (Nucleobase Ascorbate Transporter, NAT) in the accumulation of AsA has been suggested. *NAT* gene family in *Arabidopsis* and rice (Maurino et al. 2006) and in tomato (Cai et al. 2014) has been identified. Recently, a chloroplast-localized ascorbate transporter (PHT4:4), a phosphate transporter family member, has been identified for the movement of ascorbate into the chloroplasts (Miyaji et al. 2015; Fernie and Tóth 2015). A long-distance transport of AsA via phloem from leaves (source) to fruit (sink) has been demonstrated (Franceschi and Tarlyn 2002). The last enzyme, GLDH, is localized on the inner mitochondrial membrane and the supply of the direct precursor,

L-galactono-1,4 lactone to mitochondria limits AsA production and accumulation. The tagging of GLDH gene with mitochondria transit peptide for efficiently deliver of the gene product/enzyme into mitochondria and increase in the concentration of L-galactono-1,4 lactone will improve AsA production.

8.3.7 Inhibiting Ascorbate Degradation or Reducing Catabolism

Degradation of ascorbate or DHA is non-irreversible which affects their accumulation; hence, engineering inhibition of their catabolism would stabilize or increase ascorbate content. In plants, in vivo degradation of ascorbate/DHA involves enzymatic or non-enzymatic steps. The end products of the catabolism are species specific and include L-tartrate or oxalate and L-threonate. DHA is also hydrolyzed to 2,3-diketo-L-gulonate (DKG). Ascorbate and compound 1 are oxidized with ascorbate oxidase (AO). *Arabidopsis* T-DNA inserted mutants with reduced AO activity, and antisense suppression of tobacco AO has improved salt tolerance (Yamamoto et al. 2005). AO overexpressing lines exhibit complete oxidation of cell wall localized AsA pool resulting into enhanced sensitivity to ozone (Sanmartin et al. 2003) and reduced stomatal aperture, and rate of leaf water loss (Pignocchi et al. 2003; Fotopoulos et al. 2008). Decrease in AO has a role during stresses (e.g., ozone, drought, salt, and pathogen challenge); therefore, it may be vital for plant growth under natural environments (Yamamoto et al. 2005; Fotopoulos et al. 2008).

Ascorbate peroxidase (APX) reduces hydrogen peroxide to water and protect plants from oxidative stress (Mittler et al. 2004). Transgenic plants expressing antisense cytosolic APX showed decrease in APX activity with increased susceptibility to ozone injury and pathogen attack (Orvar and Ellis 1997). The knockout *Arabidopsis* plants deficient in cytosolic H_2O_2 scavenging enzyme APX form APX1, and the entire chloroplastic H_2O_2-scavenging system collapses during light stress indicating role of cytosolic APX1 in cross-compartment protection of thylakoid and stromal/mitochondrial APXs. The AO and APX activities are not positively correlated with ascorbate content; therefore, suppressing their activities would improve ascorbate levels. However, further work on downregulation of AO and APX using antisense or RNAi is required to ascertain the role of ascorbate degradation or catabolism on ascorbate accumulation.

In conclusion, advances in physiology, biochemistry, and molecular biology of ascorbate synthesis in model and crop plants have led to the engineering of elevated ascorbate content to improve crop plants productivity and resistance under adverse climate change and their nutritional value for the benefits of human and animal health. Introduction of single gene encoding enzyme within ascorbate biosynthesis and recycling pathways or regulatory protein mostly under constitutive promoter has resulted in modest increase in ascorbate amount. However, bioengineering of multiple genes of these pathways would be a more effective approach for consistent improvement in

ascorbate content by several folds as has been demonstrated earlier by transient and stable coexpression of two genes, GGP and GME of Man/L-galactose pathway, and genes of biosynthetic and recycling pathways may result in large ascorbate pool. The success of genetic engineering for high ascorbate content has been restricted to crop species that are responsive to genetic transformation. The public concerns for their potential effects on health and environment also restrict their applications for commercial cultivation. The wild relatives and some non-traditional plant species like *Myrciaria dubia* (camu-camu), *Malpighia glabra* (acerola), and *Actinidia eriantha* (wild kiwi) contain very high ascorbate levels compared to staple cereal crops. The mechanism/genetic basis for such variations and their regulation are not yet clear and thus require more basic research for the better understanding of ascorbate biosynthesis. Conventional breeding based on genetic variability in ascorbate content has led to the identification of genetic markers and QTLs/genes linked with ascorbate levels. Rapid advances in next-generation sequencing and gene editing technologies could facilitate development of crops with higher ascorbate levels. Disruption of negative regulators of ascorbate biosynthesis, e.g., targeted mutagenesis of uORF that represses the translation of GDP-L-galactose phosphorylase (GGP) or a single D27E amino acid mutation in GDP-Man pyrophosphorylase (GMP) disables interaction with CSN5B preventing its degradation through CRISPR-Cas system, may result in enhanced ascorbate biosynthesis.

8.4 Vitamin E

Enhanced production can be a solution to increasing global food demand, but it has certain limitations, and hence, it will be important to augment crop nutritional qualities. Vitamin E (VTE) is a component of a balanced diet and vital for humans. It is an effective antioxidant with free radical scavenging action and prevents certain types of diseases (Ulatowski and Manor 2015). It protects plants against lipid oxidation and stress tolerance and is crucial for cell membrane stability. VTE is a group of lipid-soluble antioxidants called as tocols or tocochromanols. First isolated from wheat germ oil (Evans et al. 1936), there are four tocopherols (T, α-, β-, γ-, and δ-) and four tocotrienols (T_3, α-, β-, γ-, and δ-) having different types of isoprenoid side chain (Kamal-Eldin and Appelqvist 1996). Tocopherols have fully saturated aliphatic tails from phytyl pyrophosphate, and tocotrienols are with an unsaturated tail containing three trans double bonds derived from geranylgeranyl pyrophosphate. Different analogues can have different position and number of methyl groups in chromanol ring which forms the headgroup. Exclusively synthesized by photosynthetic organisms, they quench polyunsaturated free radicals and break the chain reaction of lipid peroxidation. α-Tocopherol is the form with the highest VTE activity (Gutierrez-Gonzalez and Garvin 2016).

8.4.1 Sources and Deficiency

Like most of the vitamins, humans are unable to synthesize VTE naturally and obtain it in adequate quantity from their diet for which plants are the primary source (Chen et al. 2006). Tocopherols are present in almost all photosynthetic plants, remarkably in vegetable oils and nuts, whereas tocotrienols are generally present in monocot seeds (Cahoon et al. 2003). Main source is plant-derived oils where total tocochromanol levels, composition, and activity are variable (Grusak and DellaPenna 1999). α-tocopherol is the most potent VTE compound, and γ-tocopherol is the main form consumed and present in most major crops (Mene-Saffrane and Pellaud 2017).

In developed countries, a major part of population exhibit plasma α-tocopherol deficiency due to VTE-deficient diets (Mene-Saffrane and Pellaud 2017). However, it is prevalent in premature babies, digestive pathology, and genetic pathology conditions. VTE deficiency is more critical in people of developing countries (Dror and Allen 2011).

The oxidative stress due to VTE deficiency makes it necessary to fine tune its quantity and quality in the human diet and similarly for plants subject to oxidative stress. Sufficient intake of VTE has also reduced the risk of non-communicable diseases in addition to its antioxidant role.

8.4.2 Functions in Plants and Animals

In plants, VTE protects against lipid oxidation and stress tolerance and plays a key role in cell membrane stability. VTE is easily oxidized because of phenolic groups, and the derivatives produced thereby are potent antioxidants (Munne-Bosch and Falk 2004). Antioxidant ability of tocopherols and tocotrienols directly quench ROS (reactive oxygen species) inhibiting its production or indirectly terminate lipid peroxidation chain reaction (Caretto et al. 2010). In particular VTE acts as the first line of defense against lipid peroxidation. VTE prevents the oxidation of phospholipids and promotes membrane repair. Tocopherols deficiency affects seed longevity and early seedling development (Sattler et al. 2004a) and impairs photo-assimilate export in several crops like maize, potato, and tomato (Almeida et al. 2016; Hofius et al. 2004).

VTE is a critical component of balanced diet for humans. Initially, it was recognized as a nutritional factor necessary for animal reproduction. Its deficiency may result in neurological and ophthalmological disorders, myopathy (children), and hemolytic anemia in premature babies. In humans, it also acts as a powerful lipid antioxidant with free radical scavenging action and protects cell membranes from the destructive effects, and several tocochromanol isoforms prevent certain types of cancers, delay brain aging, Alzheimer's disease in older patients, cardiovascular and neurological disorders, maintain blood cholesterol levels, etc. (Ulatowski and Manor 2015).

Tocopherols and tocotrienols could decrease adenosine diphosphate-induced platelet aggregation, type-2 diabetes, immune response impairment, prostate cancer, cataracts, anemia, retinopathy, cholesterol, and inflammation activities (Kannappan et al. 2012; Jiang 2014; Mathur et al. 2015) by inducing body's defenses and humoral and cell immune responses. The tocopherol transfer protein (TTP) regulates the tocopherol secretion from hepatocytes and movement of VTE between membrane vesicles in vitro and hence maintains VTE status. Role of VTE on signaling cascades, enzyme activities, and gene regulation in mammals and plants have also been reported; however, the molecular mechanism(s) in vivo are not yet revealed (Brigelius-Flohé 2009; Asensi-Fabado and Munné-Bosch 2010).

8.4.3 Biosynthesis

VTE biosynthesis pathways (Fig. 8.6) have been found to be extensively conserved among plants (DellaPenna and Mene-Saffrane 2011). The chromanol headgroup of VTE derives from Shikimate pathway and side chain (polyprenyl) from methylerythritol 4-phosphate (MEP) pathway. Homogentisate condenses with phytyl diphosphate or geranylgeranyl diphosphate (GGDP) to form tocopherols and tocotrienols, respectively (Yang et al. 2011a, b). In the 'VTE core pathway,' *p*-hydroxyphenylpyruvic acid (HPP) dioxygenase (HPPD) converts hydroxyphenylpyruvate to homogentisate, which results in headgroup synthesis. Phytyl-PP or GGDP and homogentisic acid (HGA) then produce two intermediates in tocopherol synthesis, 2-methyl-6-phytylbenzoquinol (MPBQ) and 2-methyl-6-geranylgeranylbenzoquinol (MGGBQ) by the activity of homogentisate phytyl transferases (VTE2). The MPBQ produced is methylated and/or cyclized by 2-methyl-6-phytyl-1,4-benzoquinol transferase (VTE3) and tocopherol cyclase (VTE1), respectively, and/or methylated by γ-tocopherol methyltransferase (VTE4). The committed steps in tocotrienol and tocopherols biosynthesis are condensation of GGDP and HGA by homogentisate geranylgeranyl transferase (HGGT) and the condensation of HGA and phytyldiphosphate (PDP), catalyzed by VTE2, respectively. VTE1, VTE3, and VTE4 are common enzymes involved for the synthesis of both. The different tocopherols are products of different combinations and numbers of reactions catalyzed by VTE3, VTE1, and VTE4. Their nomenclature depends on the number and position of methyl substitutions on chromanol ring (Stacey et al. 2016). In plants, tocochromanol and enzymes of the core pathways have been found in plastids only.

8.4.4 Biofortification

Biofortification is an approach to enrich nutrient(s) content to reduce micronutrient deficiencies, especially vitamins, minerals, etc., in staple crops to sustain nutritional and health goals. Identification of VTE biosynthetic genes has helped to improve the

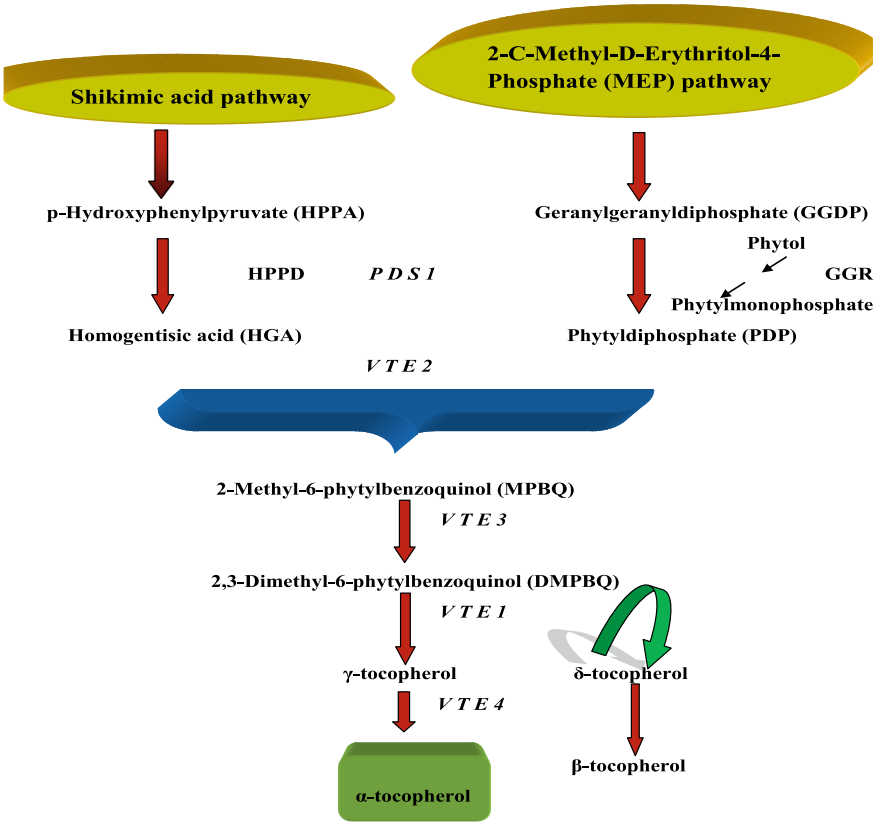

Fig. 8.6 Pathway for biosynthesis of vitamin E

VTE content of crops either by metabolic engineering or classical breeding (Garcia-Casal et al. 2017).

Recently, numerous genes for VTE pathway enzymes have been identified and cloned using genetic and genomics-based methods, but studies are still limited to the model organisms like *Arabidopsis thaliana* and *Synechocystis* (DellaPenna and Mene-Saffrane 2011; Wang et al. 2018). However, the genetic and genomics-based findings on tocopherol biosynthetic genes from *Arabidopsis* can be efficiently applied to other plants to isolate their orthologs from their genome sequences. This has enabled manipulation of tocochromanol levels, types, and accumulated end products in species like maize, soybean, canola, tomato, and others (Shukla and Mattoo 2009; Quadrana et al. 2013; Lira et al. 2016). VTE has been increased by regulating the activity of enzymes involved in tocopherol biosynthesis including HGGT (Cahoon et al. 2003), MBPQ-MT/VTE3 (Sattler et al. 2004b; Tang et al. 2016), HST (Sadre et al. 2006), HPT1/VTE2 (Seo et al. 2011), γ-TMT/VTE4 (Ghimire et al. 2011; Yabuta et al. 2013; Zhang et al. 2013), HPPD (Farre et al. 2012), and TC/VTE1 (Yabuta et al. 2013).

8.4.5 Plant Breeding/Genome-wide Association Studies

High variation found in VTE content and identification of micronutrient related QTLs in different agronomically important crops can be exploited by plant breeders for biofortification (Shammugasamy et al. 2015; Mene-Saffrane and Pellaud 2017). Genome-wide association studies (GWAS) help to distinguish the natural allelic variations controlling VTE. Functional genomics studies in modal organisms can be potentially applied to important crop plants. Quantitative trait loci (QTLs) controlling the accumulation of VTE have been identified in *Arabidopsis*, sunflower, maize, and tomato (Gilliland et al. 2006; Hass et al. 2006; Schauer et al. 2006; Chander et al. 2008; Almeida et al. 2011). Association mapping approach is now also used to discover new alleles (Myles et al. 2009).

Recent joint-linkage mapping and GWAS related to natural VTE level variations helped to understand the genetic control of tocochromanols in maize. Out of 52 QTLs identified for individual and total tocochromanols, 14 resolved to individual genes (responsible for 56–93% of phenotypic variation), 6 encoded new activities and included two chlorophyll biosynthetic enzymes (por homologues) explaining the majority of tocopherol variations. Based on detailed studies, the authors suggested that total tocopherols (two por homologues), total tocotrienols (*hggt1, hppd1, and dxs2*), or VTE content (*vte3 and vte4*) can be targeted further by genomics-assisted breeding approaches either separately or in combinatorial fashion for fine-tuning of various essential micronutrients (Diepenbrock et al. 2017).

Identification of a gene encoding 2-methyl-6-phytylbenzoquinol methyltransferase of the *Arabidopsis* mutation *vitamin E pathway gene 3-1* (*vte3-1*) and its seed-specific expression in transgenic soybean reduced seed tocopherol from 20 to 2% (Van Eenennaam et al. 2003). These results confirm that transgenic expression of *VTE3* from *a* model organism controlled by a seed-specific promoter alters soybean tocopherol composition for nutritional and food quality implications. Coexpression of *At-VTE3* with *At-VTE4* in soybean resulted in more than eightfold tocopherol and fivefold VTE activity in seeds. Overexpression of the *γ-TMT*-(γ-tocopherol methyltransferase) gene in *Arabidopsis* seeds increased the tocopherol composition. The VTE activity was increased ninefold, thereby increasing the nutritional value (Shintani and DellaPenna 1998).

A short interspersed nuclear elements (SINE) retrotransposon in the promoter region of VTE3 increased VTE accumulation in tomato (Quadrana et al. 2014). In maize, three genes, *VTE1, HGGT1*, and a prephenate dehydratase paralog, were responsible for tocotrienol variations (Lipka et al. 2013) and similarly, two insertion/deletions within VTE4 and a single-nucleotide polymorphism (SNP) varied α-tocopherol contents (Li et al. 2012a, b). GWAS established that natural tocopherol variations in maize kernels are affected by genes involved in fatty acid and chlorophyll metabolisms and functions of chloroplast. Forty-one unique QTLs and 32 significant loci were identified in 6 populations of recombinant inbred lines (RILs). The study was validated by the fine mapping of a major QTL and suggested that

non-tocopherol pathway genes are also responsible for natural tocopherol variations (Wang et al. 2018).

The coding sequences of genes of the VTE pathway have been elucidated, including individual homeologs to understand oat genome evolution and natural variation for VTE (Gutierrez-Gonzalez and Garvin 2016). The study highlights the role of VTE biosynthetic genes expression in observed composition of VTE forms in oat seeds. The expression of HPPD, homeologs of VTE2 and VTE4 and VTE were highly correlated. The findings are helpful to modify VTE content and composition in oats and to design markers for key genes involved in VTE accumulation.

8.4.6 Metabolic Engineering

Metabolic engineering has gained a lot in recent times by expansion of fundamental knowledge of plant metabolism (Strobbe and Van Der Straeten 2018). Conventional breeding is widely used but has some serious issues like sexual incompatibility, linkage drag, and lengthy breeding programs. However, genetic engineering gives advantage of directly introducing new genes from any source into local varieties using different approaches to generate various phytonutrient improved crops. Single or multiple genes have been transferred for biofortification of vitamins in several crops.

Homogentisate phytyltransferase (HPT) enzyme is involved in the biosynthesis of tocopherols, and ectopic expression of its homologue gene, *MdHPT1*, isolated from apple in tomato plants has resulted in significant elevated levels of α-tocopherol in transgenic leaves and fruits (Seo et al. 2011). Introduction of barley *HGGT* gene in maize has resulted in sixfold increase in the total tocotrienols and tocopherols (Cahoon et al. 2003). Overexpression of *γ-TMT* in canola (Van Eenennaam et al. 2003) and soybean (Sattler et al. 2004a) has improved α-tocopherol by seven times. Similar approach has also increased α-tocopherol by about two times in lettuce (Cho et al. 2005) and *Perilla frutescens* (Ghimire et al. 2011). Overexpression of key plastid-localized enzymes (HPT1/VTE2, TC/VTE1, and γ-TMT) in tobacco and tomato plants has resulted in ten times higher accumulation of total tocochromanol (Lu et al. 2013).

Tocopherol contents are correlated with carotenoid biosynthesis and chlorophyll metabolism. Phytoene synthase (PSY), a core biosynthetic enzyme in carotenoid biosynthesis, on overexpression in tomato, has increased tocopherol levels (Fraser et al. 2007). Similarly, independent overexpression of a bacterial phytoene desaturase has also resulted in higher tocopherol content in fruit (Römer et al. 2000). Chlorophyll synthase catalyzes the esterification of chlorophyllide with either GGDP or phytyl diphosphate (PDP). Suppression of chlorophyll synthase (*CHLSYN*) by RNAi has reduced chlorophyll accumulation but increased tocopherol concentrations by two- to threefold, whereas its overexpression has resulted decrease in tocopherol concentrations in leaves of *A. thaliana* (Zhang et al. 2015a, b).

Coexpression of VTE through ectopic expression of *HGGT* stabilizes provitamin A in sorghum either by preventing β-carotene oxidation during storage or increasing the efficiency of vitamin A conversion by promoting cleavage of the β-carotene molecule during consumption (Che et al. 2016). Some other studies have shown that a combined expression of more than one gene of a biosynthetic pathway has resulted increase in overall expressions. VTE3 and VTE4 control the different tocopherol ratios (Quadrana et al. 2014). Increasing expression of *VTE3* and *VTE4* converts the tocopherols in seed to α-tocopherol (Van Eenennaam et al. 2003). *HPT* and *γ-TMT* together has resulted in a 12-times higher VTE in *Arabidopsis* (Collakova and DellaPenna 2003). Similarly, *MT* and *γ-TMT* have increased VTE by five times (Van Eenennaam et al. 2003; Sattler et al. 2004a), and *tyrA* (HPT in yeast) and *Arabidopsis HPPD* and *HPT* have increased VTE in soybean by 11 times (Karunanandaa et al. 2005). However, tocopherol biosynthetic pathway is complex and yet partially understood (Zhang et al. 2014; Lin et al. 2016), and additional genes are still getting identified (Hey et al. 2017; Liao et al. 2018; Pellaud et al. 2018).

In conclusion, although, understanding of the plant biosynthetic pathways for VTE has significantly increased in recent times, still limited number of genes has been explored for biofortification. Overexpression of multiple genes involved in VTE biosynthesis is a promising strategy. Besides, the main goal of VTE biofortification is to increase its contents which can be achieved by converting all the other derivatives into α-tocopherol as it has the highest biological activity. Homeolog-specific sequence variations and differential expressions help to design markers for key genes regulating VTE accumulation. Advanced genomic studies for understanding chlorophyll turnover will enhance VTE biofortification as phytyl pyrophosphate (from which fully saturated aliphatic tails of tocopherols are derived) mostly comes from the recycling of chlorophylls. A combined approach to exploit natural genetic variability of a given trait using molecular markers and engineering vitamin pathways or vitamin-related metabolism should be used as a complementary to each other.

8.5 Challenges, Opportunities, and Conclusions

Over the last few decades, significant advances in physiology, biochemistry, and molecular biology of vitamins especially their biosynthetic pathways and regulation in model and crop plants have paved the way for engineering of elevated vitamin(s) content to improve their productivity, stress resistance, and nutritional value for the benefits of human and animal health. However, still limited numbers of genes have been overexpressed (mostly single) in crop plants to increase their vitamin content. Engineering multiple genes of a vitamin biosynthetic pathway or of more than one vitamin (multi-biofortification) in a local elite variety would be more effective to overcome vitamin(s) deficiencies. Biofortification approaches should take into account prevention of vitamin losses during post-harvest storage, processing and cooking, promotion of transport and storage stability of vitamins in the edible parts,

absorption after consumption (bioavailability), and their efficacy to improve vitamin status without affecting yield and other farmer/consumer preference traits. Conventional breeding based on genetic variability has led to the identification of genetic markers and QTLs/genes required for more efficient breeding of varieties with improved vitamin content. Conventional breeding and metabolic engineering of vitamin pathways or vitamin-related metabolism should be used as a complementary to each other. Rapid advances in genomics and next-generation sequencing, metabolomics, and GWAS would identify elite allelic variations, intermediate metabolites, and rate-limiting steps, and would help to design novel genes and metabolic pathways. Further, use of genome-editing techniques such as the CRISPR/Cas can ease expensive and stringent regulatory issues and will improve public acceptance of vitamin-enriched crops for the nutritional well-being of poor people. Collaboration between scientists, nutritionists, policy executioners, funding agencies, and educators and further promotion of nutrition-sensitive agriculture and food-based strategies must be adopted to produce staple food crops with sufficient levels of all vitamins to resolve the problem of vitamin deficiencies globally.

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Chapter 9
Strategies that Influence the Production of Secondary Metabolites in Plants

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Abstract Challenge of today's agriculture requires the innovative application of techniques and methodologies to increase the production of high nutritional quality food crops with greater vigor and more productivity. In the previous decades, consumers have been looking for foods that not only provide the nutrients (lipids, carbohydrates, and proteins) but also the compounds with health benefits, such as carotenoids, flavonoids, phenols; these are not part of the plant's primary metabolism but provide protection, attraction, survival, aroma, color, flavor, etc. The production of several of these compounds, known as secondary metabolites, is influenced by a wide range of factors such as biotic and abiotic stresses, types of fertilization, agronomic management, elicitors, and, recently, the presence of nanoparticles, without neglecting the use of biostimulators and biocontrollers, in addition to metabolic engineering manipulation. These factors influence both positively and negatively the production of secondary metabolites, giving the plant strategies for its adaptation and survival, as well as compounds with biological activity that contribute to the health of the human being. In this sense, the present proposal intends to gather relevant and current information on how some of these strategies influence the production of secondary metabolites in plants.

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9.1 Introduction

The plants in their environment are continuously modifying their metabolism, because they are subject to environmental changes as well as the attacks of pathogens and pests (Cramer et al. 2011). If changes in the crops are positive, they are called eustressors; otherwise, they are called distressors. When plants are stressed, they stimulate the secondary metabolism for the production of substances that help them to cope up with the induced stress (Jamwal et al. 2018). These synthesized substances are called secondary metabolites (SMs) (Kacienė et al. 2015; Neugart et al. 2018). The SMs confer to each plant the particular characteristics of flavor, aroma, color, nutrients, medicinal properties, and the protection against stress (Gorelick and Bernstein 2014; Lajayer et al. 2017a; Pavarini et al. 2012). Nowadays, farmers are using techniques involving stress factors to accelerate the production of these plant compounds, intensifying flavors, colors, promoter of growth, increasing nutraceutical quality, and even lengthening the shelf life (Guo et al. 2015; Lajayer et al. 2017b; Le Mire et al. 2016). This chapter collects relevant and current information on the various techniques that are being used, such as metabolic engineering, nanostructures and/or nanomaterials, biostimulators, biocontrollers, and elicitation to improve the production of SMs of agronomic and nutraceutical interest.

9.2 Elicitors

Plants own a vast range of defenses that could be produced not only in response of parasites or pathogens but also in recognition of beneficial saprophytic microorganisms. Compounds derived from these microorganisms or from the plant itself are perceived by the plant to activate a local or systemic resistance. Also, abiotic stress such as inorganic salts, metal ions, chilling, extreme temperatures, and wounding can induce a resistance response (Gorelick and Bernstein 2014). These abiotic and biotic conditions that induce plant stress are called elicitors. The stimulus of pathogen response and plant gene regulators can also be attained through biostimulants (chitosan, laminarin, etc.) that contain substances which act as elicitors (du Jardin 2015). The focus of this section is on biotic and abiotic stresses and the substances that mimic these stress conditions that have an impact on the production of plant metabolites.

Several plant metabolites are used as herbicides, pigments, pharmaceuticals, fragrances, etc., because they are the major sources of important bioactive compounds. Research has been done on methods to increase plant products with beneficial properties. A vast array of these benefits comes from SMs, i.e., chemicals produced by plants that are not essential for the normal plant growth, development, or reproduc-

tion, but that are implicated in plant defense against herbivory and other interspecies defenses. Elicitors act as switch for stimulating the accumulation of SMs in plants and plant cell cultures (Gorelick and Bernstein 2014; Namdeo 2007; Shilpa et al. 2010).

Elicitors could be classified as general or specific according to their mode of action. While general elicitors are involved in primary innate immunity and are able to trigger defense both in host and non-host plants and do not deeply diverge in their effects on different cultivars in a plant species, specific elicitors are compounds in certain pathogens and function only in plant cultivars that match the corresponding disease resistance gene and usually lead to the secondary innate immunity after an intracellular receptor-mediated perception. General elicitors include chemicals, microbe-associated molecular patterns (MAMPs) from non-pathogenic microorganisms, damage-associated molecular patterns (DAMPs) from plant surfaces resulting from the action of the invading agent, and pathogen-associated molecular patterns (PAMPs) from pathogenic microorganisms (Bent and Mackey 2007; Henry et al. 2012). On the basis of their origin, elicitors could be either biotic or abiotic.

9.2.1 Biotic Elicitors

Biotic elicitors are biological molecules of either pathogen or host origin that can induce defense responses which may be released from the attacked plant by enzymes belonging to the pathogens (e.g., jasmonic acid, salicylic acid, etc.). Biological mixtures prepared from microorganisms (pathogenic or non-pathogenic) have been used in different cultivars and cells cultures to induce the production of different metabolites. Often, the molecular structure of the active ingredients in these complex biological preparations is unknown (Gorelick and Bernstein 2014; Rao and Ravishankar 2002). Purification of these biological mixtures has led to the description of many diverse biotic elicitors which include lipopolysaccharides, polysaccharides (e.g., pectin, cellulose, chitin, alginate, carrageenan), oligosaccharides (e.g., galacturonides, mannan, etc.), proteins, and pathogen toxins (Bi et al. 2011; Gururaj et al. 2012; Montesano et al. 2003; Ryan 2000).

Elicitors can be obtained from fungus, bacteria, and yeast. Yeast and fungal extracts (*Aspergillus niger* and *Penicillium notatum*) were applied in *Psoralea corylifolia* cell cultures to improve accumulation of psoralen. Psoralen is a furanocoumarin that exerts a range of pharmacological activities. The results showed that cell treated with *A. niger* elicitor exhibited an increase of ninefold in the concentration of psoralen compared to control cells. Cells treated with *P. notatum* and yeast extract had an increase of four- to sevenfold psoralens over control cells. The authors indicated that the elicitor used at the 0.5–3% v/v concentrations improved the accumulation of psoralen, but the *A. niger* elicitor at 1.0% v/v induced the greatest accumulation

(Ahmed and Baig 2014). El-Nabarawy et al. (2015) applied mevalonic acid, different amino acids (Phe, Leu, and Val) as well as yeast extract and a fungal extract (*Aspergillus niger*) to ginger to produce gingerols and shogaols. Gingerol, a compound with great potent antioxidant activity, increased with the addition of mevalonic acid and yeast extract in callus cultures. Yeast extract was also applied in *Oldenlandia umbellata* root cultures to improve anthraquinones. Results showed that the production increased significantly at elicitor concentrations of 25 and 50 mg/L. Grapes varieties, Monastrell and Tempranillo, treated with yeast cell wall exhibited higher levels of stilbene compared with control grapes. Ajmalicine accumulation increased by about threefold when cells of *Catharanthus roseus* were treated with the fragments of fungal cell wall (*F. moniliforme*, *T. viride* and *A. niger*). Cells treated with *T. viride* showed the highest ajmalicine production (Namdeo et al. 2002).

Yeast and fungal extracts contain amino acids, vitamins, and minerals. It has been stated that their elicitation effect is related to the presence of Zn, Ca, and Co, cellulose, chitin, lipids, sterols, and proteins, as well as their role in increasing the phenylalanine ammonia lyase activity (PAL), a key enzyme of phenylpropanoid pathway that connects primary metabolism to the secondary one, and is implicated in the production of phenylpropanoids (Kapteyn et al. 1999; Kozarski et al. 2014).

Plant hormones (e.g., auxins, gibberellins, cytokinins, abscisic acid, ethylene, etc.) have a decisive role in plant biology as they regulate a vast variety of plant characteristics. Like hormones, salicylic acid and jasmonates trigger cellular responses at low concentrations far away from their site of synthesis and can be administered to plants in a many ways. Salicylic acid and jasmonates are well known to elicit an array of substances through the expression of plant genes implicated in various biosynthetic pathways (Wasternack and Hause 2013). Brassinosteroids, one of the last hormones discovered, have also been suggested to have a role in abiotic stress responses (Rao et al. 2002). Application of commercial yeast extract, methyl jasmonate, and chitosan on grapes increased the anthocyanin content of grape and wine (Portu et al. 2016). Methyl jasmonate and salicylic acid have been found to be beneficial in improving major carotenoids and α-tocopherol in the foliage of *Moringa oleifera* (Saini et al. 2016). Salicylic acid application has increased the concentration of phenolic compounds and antioxidant activity in peppermint. Ethylacetate and methyl jasmonate increased saponin content in soybean variety Ozark (Eswaranandam et al. 2012). The impact of phytohormones and signaling molecules (e.g., reactive oxygen species (ROS)) on plant development and metabolism has been extensively reviewed earlier (Ashraf et al. 2010; Dar et al. 2015; Hung et al. 2005; Rivas-San Vicente and Plasencia 2011; Wasternack and Hause 2013).

9.2.2 Abiotic Elicitors

Abiotic elicitors can be of chemical (inorganic salts, metal ions, and others which disturb the membrane integrity) and physical (UV irradiation, wounding, saline stress, ozone, etc.) origin. This type of elicitors refers to factors related to environmental

stress. Capanoglu (2010) studied the antioxidant activity (ascorbic acid, tocopherol, and phenolic contents) of different crops (potatoes, celery, strawberry, carrot, maize, lettuce, spearmint, tomato, peanut, apple, apricot, mango, onion, arugula, broccoli, beans) under various abiotic stresses during pre-harvest and post-harvest. The increase in these compounds highlights the use of elicitors in food quality enhancement. In the same way, the effects of abiotic environmental stresses on crop quality are summarized by Wang and Frei (2011); their work presents some general trends in crops such as increase in antioxidants and proteins and/or decrease in lipid and starch concentrations. The information presented is useful in developing strategies to improve the quality of crops but should be carefully analyzed since it depends on many concurrent physiological, environmental, and experimental factors. Reports sometimes seem contradictory, even if the same stress type and species were investigated.

UV-B treatment at post-harvest in peach and nectarine fruits is an effective tool to induce metabolite production (Scattino et al. 2014). Flavonoids, glucosinolates, ascorbic acid, and carotenoids appear to be stimulated in different crops by water stress (Stefanelli et al. 2010). Ginseng cells on exposure to an ultrasonic treatment (38.5 kHz, 810 W) in a ultrasonicator (bath type) have increased their ginsenoside saponins nearly by 75% (Lin et al. 2001). Seeds treated with magnetic fields have displayed a higher production of ROS and an activation of antioxidant defense system, suggesting that magnetic fields can stimulate the stress responses of plants and seeds (Dannehl 2018). Gamma irradiation had a stimulatory effect on the production of naphthodianthrones and phenolic compounds in *Hypericum triquetrifolium*. Further, irradiation with 10 Gy exhibited the maximum amounts of hypericin and pseudohypericin. Ozone concentration at 5 mL/L for 48 h is found to be optimum to induce a twofold increase in the phenolics, flavonoids, and water-soluble polysaccharides in *Ganoderma lucidum* (Sudheer et al. 2016). Non-thermal technologies (pulse electric fields, ultrasound, and high pressure processing) are reported to induce immediate and late stress responses similar to wounding stress. Although additional investigations should elucidate optimum non-thermal processing conditions that induce the highest accumulation of nutraceuticals in horticultural crops, it has been suggested that these technologies may be used as an elicitation technique to produce functional foods (Table 9.1).

Table 9.1 Classification of elicitors

Abiotic	Chemical	Inorganic salts
		Metal ions
	Physical	Wounding
		Saline stress
		UV irradiation
		Ozone
		Acoustic waves
		High pressure
		Extreme temperature
		Modified gas composition
Biotic	Complex composition	Yeast, fungal extracts, bacteria
	Defined composition	Liposaccharides
		Polysaccharides
		Oligosaccharides
		Proteins
		Pathogen toxins

9.2.3 Mode of Action

Elicitation enhances SMs in plants or plant cells in vitro, but the detailed mechanism of elicitation is not fully understood. The exact mechanism of elicitor perception is beyond the scope of this chapter, but in general, the biosynthesis of plant products involves the perception of an extracellular or intracellular signal by a receptor located in the plasma membrane; then, a signal transduction cascade is triggered to activate the de novo biosynthesis of transcription factors that lead to the expression of genes implicated in secondary metabolism. When a plant or plant cell culture is challenged with a elicitor, a series of biochemical activities takes place; some of them are the generation of ROS, accumulation of proteins related to pathogenesis such as chitinases and glucanases, hypersensitive response (cell death at the site of infection), structural changes in the cell, defense response genes, synthesis of defensive molecules of plants such as tannins, synthesis of jasmonic and salicylic acids as secondary messengers, and finally, the acquired systemic resistance (Baenas et al. 2014).

The mechanisms by which elicitors could be recognized at the plasma membrane level are described by Boller and Felix (2009) and Henry et al. (2012). Evidence for a regulatory cross talk between signaling pathways (salicylic and jasmonic acid and ethylene) that takes part in plant defense against pathogens is provided by Kunkel and Brooks (2002). In the previous years, more plant receptors for elicitors have been identified; Zipfel (2014) has summarized several molecular strategies employed by plant pattern-recognition receptors to stimulate innate immune signaling for survival. Elicitors related to herbivores and their signaling mechanisms working in plants after their perception have been reviewed by Bonaventure et al. (2011).

There is no way to predict if an elicitor will be effective in a plant or specific cell system for metabolite accumulation. A great amount of research has been done to study the effect of elicitors. Table 9.2 presents some of the researches carried out on this topic. To improve the quality of crop plants, it is necessary to get a full understanding of the stress conditions that enhance the accumulation of the

Table 9.2 Plant elicitation

Plant species	Product	Elicitor	References
Oldenlandia umbellate	Anthraquinones	Yeast extract, pectin, xylan	Krishnan and Siril (2018)
Lactuca sativa	Phenols	Chitosan and tea tree essential oil	Viacava et al. (2018)
Zingiber officinale Rosc.	Phenolic and flavonoid contents	Yeast extract and salicylic acid	Ali et al. (2018)
Ocimum basilicum	Phenylpropanoids, terpenes	$CuSO_4$	Trettel et al. (2018)
Chlorophytum borivilianum	Diosgenin	Jasmonic and salicylic acid	Chauhan et al. (2018)
Salvia miltiorrhiza and *Salvia castanea*	Tanshinone	Yeast extract and Ag^+	Yang et al. (2018)
Centella asiatica	Asiaticoside without a triterpenoid	*Piriformospora indica*	Jisha et al. (2018)
Fagopyrum esculentum M	Tocopherols, β-carotene, flavonoids	Sucrose	Jeong et al. (2018)
Isatis tinctoria	Flavonoids	*A. niger*	Jiao et al. (2018)
Salvia officinalis	Flavonoids, terpenes	Chitosan, drought stress	Vosoughi et al. (2018)
Ocimum basilicum	Phenols	UV-B	Mosadegh et al. (2018)
Eucalyptus globulus	Isoprene and mono- and sesquiterpene saturated aldehydes (C7-C10), benzenoids	Ozone and wounding	Kanagendran et al. (2018)

target compounds. The understanding of physiological and biological basis of the induction of plant metabolites has greatly advanced over the past years, but a deeper investigation of the mechanisms underlying the perception of elicitors is essential to tailor the production of specific metabolites.

9.3 Biostimulators

Horticulture specialists define biostimulator as a material which in small quantities promote plant growth without nutrients, soil improvers, or pesticides. Some of these biostimulators are humic acids, seaweed extracts, chitosan, biopolymers, inorganic compounds, and beneficial fungal and bacterial formulations (Kauffman et al. 2007). On the other hand, whether the crop's endogenous antioxidant activity under stress is approachable by the exogenous hormone may be considered metabolic enhancers as well as biostimulant (Zhang et al. 2003). The cytokinins and auxins or their by-products are substances which activate plant growth (Calvo et al. 2014). Thereby, the positive actions of natural or synthetic biostimulants produce chemical changes within the crops that cause growth promotion, resistance to abiotic or biotic stress, and modulation of development. Such changes can be attributed to bacteria and fungi (du Jardin 2015). The beneficial fungi and bacteria integrate groups or categories with biostimulative effect. These categories are briefly presented in Table 9.3.

9.3.1 Classification of Plant Biostimulants

9.3.1.1 Fulvic Acids

Compost is formed by the microbial decomposition of organic waste, and the microbes transform the organic substrate to the soil organic matter called humic substances. Though soluble humic and fulvic acids have a positive effect on plant growth, that may be inconsistent in some plants. Humic substances from composts and vermin composts have been used for many years as natural fertilizers in the soil due to their positive effect on the physicochemical and biological properties of soil as well as on plants (du Jardin 2012). The biostimulant effects of the humic substances are related to the absorption of macro- and micronutrients, the amelioration of root nutrition, and the exchange of positive soil radicals, containing the polycations (Jindo et al. 2012). Also, the invertase of the substrate C increases and the breathing improves due to the humic substances. The high molecular humic substances have shown that secondary metabolism and the response of the plants to the biotic and abiotic stress in the hydroponically cultivated corn plants improve the enzymatic

Table 9.3 Effects of biostimulants in crop productions

Biostimulators	Effects in crop productions			Cellular mechanism	References
	Crop performance	Changes in yield	Physiological function		
Humic and fulvic acids	Enhanced nutrient use efficiency	Saving of fertilizers	Root biomass	Activate ATPases in maize roots	Jindo et al. (2012)
Protein hydrolysate	Tolerance to abiotic conditions	Yield under abiotic stress	Protection against weather and oxidative damage	Activation enzymatic and PAL and production of flavonoids in abiotic stress	Ertani et al. (2013)
Seaweed extracts	In plant tissues improved mineral composition (S, Fe, Zn, Mg, Cu)	Biofortification of plant tissues	The transport of micronutrients increases in the tissue concentrations	*Ascophyllum nodosum and Brassica napus* stimulate expression of genes	Battacharyya et al. (2015)
Chitosan	Plants against fungal pathogens, abiotic stress tolerance	Yield under abiotic stress	Stomatal closure induced by chitosan through a mechanism dependent on ABA	The accumulation of hydrogen peroxide and the Ca^{2+} filtration to the cell are keys in the signaling of stress	Iriti et al. (2009)
Other biopolymers	Tolerance to abiotic stress	Yield in salinity conditions	The crop under salt stress maintenance photosynthesis	Protects photodamage and likely pathway activation ROS	Chen and Murata (2011), Shabala et al. (2012)
Inorganic compound	Attack of pathogens for selenium and osmotic stress for sodium	Positive effects on the growth of plants and the response to stress	Strengthening of cell walls by siliceous deposits	Promote the growth of plants as inorganic salts and amorphous silica	Pilon-Smits et al. (2009)

(continued)

Table 9.3 (continued)

Biostimulators	Effects in crop productions			Cellular mechanism	References
	Crop performance	Changes in yield	Physiological function		
Beneficial fungi	Many plant responses are also induced, including a greater sufferance to abiotic stress	Reduction of fertilizer use	Nutrition efficiency, water balance, biotic and abiotic stress protection of crop	The fungal conduits allow for interplant signaling	Behie and Bidochka (2014)
Beneficial bacteria	Use efficiency by enhanced nutrient and increased root	Saving of fertilizers	Increases resistance in the root by morphological changes	Plant growth-promoting Rhizobacteria that activates signaling pathways	Ahmad et al. (2008a, b)
Phosphite	Biomass dry weight, foliar area, early growth	Yield in percentage of jumbo size onions, potatoes, peppers	Protection against UV-B and activation of the antioxidant system	Reinforcement of the cell wall and defense response emergence, mycorrhizal colonization	Gómez-Merino and Trejo-Téllez (2015)

activity for the production of phenolic compounds, suggesting modulation of stress responses (Olivares et al. 2015). ATP hydrolysis into a transmembrane depicts an increase in the absorption of nitrate and other nutrients. So this is another contribution of humic substances to root sustenance as a stimulus H^+-ATPases of plasma membrane (Schiavon et al. 2010).

9.3.1.2 N-Containing Compounds

The organic compounds on hydrolysis produce amino acids as well as betaines, polyamines, and "non-protein amino acids" mixtures, with well-known antistress properties (Chen and Murata 2011). These compounds act as biostimulants of plant growth by compounds containing N and also produces structural changes at the genetic level by the absorption of N in the roots of plants (Calvo et al. 2014), as well as the scavenging of free radicals which help in the path of the environmental stress in the plant, likely, roots have the function of absorbing nutrients increasing biomass, soil respiration and also works as fertilizer (Colla et al. 2014). When analyzing hydrolyzed proteins of animal origin, these were considered safe since they do not generate

toxicity on experiments with yeasts and plants as assessment material. However, it is not recommended to use protein hydrolysates from animal by-products (Corte et al. 2014).

9.3.1.3 Seaweed Extracts

The seaweed extracts contain compounds like chitosan which are applied to root or foliar of plants at different stages of growth (Haider et al. 2012). Other used seaweed extracts could be the polysaccharides laminarin, alginates, and carrageenans and their breakdown products (Craigie 2011). The brown algae species such as *Ascophyllum*, *Fucus*, and *Laminar* are used as biostimulators for the improvement in plant development (Khan et al. 2009). Seaweed extracts applied on foliar or in hydroponic solution can be used to capture heavy metals, induce bioremediations to achieve greater production of bacteria, and have an effect on growth as a fertilizer to improve seed germination and activation of hormones for plant development. Some hormone-like compounds, sterols and polyamines, have antioxidant effects and regulate endogenous stress and biosynthetic genes of plant tissue hormones (Halpern et al. 2015).

9.3.1.4 Other Biopolymers

The chitosan oligomers have the ability to capture polycationic compounds and also bind to a broad range of cellular compounds including DNA, plasma membrane, and cell wall and can protect the plant cell (Hadwiger 2013; Katiyar et al. 2015). Various receptors and signaling pathways for binding of chitosan have demonstrated the accumulation of hydrogen peroxide and Ca^{2+}, the fundamental performers in the signaling of stress responses and their regulation (Ferri et al. 2014).

9.3.1.5 Inorganic Compounds

Aluminum (Al), cobalt (Co), sodium (Na), selenium (Se), and silica (Si) are elements that promote crop growth and may be essential to specific taxa (Pilon-Smits et al. 2009). These elements have different plant-related activities such as plant hormone synthesis and signaling, better nutrition through interaction with other elements during absorption and mobility, rigidity in the cell wall, impact on pathogen attack, osmoregulation, enzyme activity by cofactors, reduced transpiration of crystal deposits, and many other benefits for the plants (Povero et al. 2011).

9.3.1.6 Beneficial Microorganisms

The beneficial microorganisms interact with plants in all ways. The fungi could have an interaction through mutualism with crop roots and parasitism. The effects of bios-

timulators in plants that have beneficial microorganisms are very important since they have the functions of inducing the resistance to diseases and control of the morphology as they are regulating the growth, enhancement of abiotic stress tolerance, supply of nutrients, and increase in the use of nutrients (Augé 2001; Gianinazzi et al. 2010; Siddiqui and Pichtel 2008; Van Der Heijden et al. 2006).

9.3.1.7 Phosphite

Celery, onion, potato, and pepper are some of the crops that have shown a positive effect on the use of phosphites principally in terms of yield, produce quality, and allowance to abiotic stress issues. For example, in celery, the yield was significantly increased, when phosphite used in hydroponic or foliar application showed increased percentage of oversized onions and potatoes. Applying phosphite in the form of foliar spray or drip on sweet pepper showed a significant increase in yield. Phosphite fertilization in this way has a greater effect on the yield; therefore, it is better to absorb the phosphorous derivative of phosphite than inorganic phosphate (Pi) (Thao and Yamakawa 2009). Phosphite (Phi) is not a good source of phosphorous (P) for *Brassica napus* cultured cells and *Brassica nigra* plantlets (Carswell et al. 1997), as well as for hydroponically grown tomato and pepper (Förster et al. 1998). The plants treated with phosphite showed changes in glycolysis and hormonal levels. It is also believed to induce the shikimic acid pathway, so all this depicts an increase in flowering but not in the content of soluble solids in different crops. In lettuce, tomato, and banana, the use of Pi plus Phi (50% as HPO_4^{-2} and 50% as $H_2PO_3^{-}$) in a hydroponic system enhanced the biomass dry weight, foliar area, and P content in the whole plant (Bertsch et al. 2009). Lovatt (1990) revealed that foliar application of potassium-Phi (K_3PO_3) to P-deficient citrus seedlings influenced biochemical changes similar to those of calcium phosphate application and also re-established plant growth. Additionally, Lovatt (1998) showed that foliar spray of K_3PO_3 to navel orange trees considerably increased the number of commercially valuable overgrown size fruit, improving the ratio of total soluble solids and acidity, as compared to control fruits (Lovatt 1998). Similarly, Albrigo (1999) informed that foliar applications of Phi to Valencia oranges increased flower number, fruit set, and yield, along with total soluble solids.

9.3.2 Biostimulation Proficiency on Plant Growth

The substances that generate an improvement in crops such as macro- and micronutrient content, vitamins, gibberellins, cytokinins, and auxins are biostimulators. These are considered valuable compounds for greater yield in plants (Stirk and Van Staden 1996). Some authors, with the use of these biostimulators, have managed to increase the yield of the agricultural crops and greater fruit size in citrus, cucurbits, and tomato. Therefore, the positive effects of biostimulators are due to the higher quantity of sec-

ondary metabolites which counteract pathogens (Michalak and Chojnacka 2015). For example, the methanolic extract of *Sargassum wightii* (*S. swartzii*) and *C. agardh* has shown the highest activity against the phytopathogenic bacterium *Pseudomonas syringae* which causes leaf spot disease on the treasured medicinal plant *Gymnema sylvestre* (Kumar et al. 2008). *Sargassum polyceratium*, *Caulerpa racemosa*, and *Gracilaria cervicornis* have active effects against *Staphylococcus aureus* (Borbón et al. 2012). *S. swartzii* has been identified to inhibit the growth of *Xanthomonas oryzae pv. oryzae* which causes bacterial blight of rice (Hamed et al. 2018). Moreover, *Cystoseira stricta* extracts defend plants against bacteria due to the high content of phenolic compounds (Lee and Jeon 2013). Marine macroalgae extracted polysaccharides can be used in the plant defense against some infectious pathogens. These algae contain chlorophyll, alginates, furans, laminarin, carrageenan, and porphyrins (Hamed et al. 2018).

9.4 Biocontrollers

Crop plants are exposed to various insects, parasites, viruses, and/or pathogens that compete for space and nutrients resulting in economic losses equivalent to billions of dollars (Mishra and Arora 2018). In 2015, the Food and Agriculture Organization (FAO) estimated that about 25% of crops loss globally is by pests and diseases (FAO 2015). This has led to the indiscriminate use of herbicides, insecticides, and pesticides resulting into development of the resistance in pathogens, and in several cases, the accumulation of their residues in crop plants not only affects the yield of the product but also causes harm to human health (Aktar et al. 2009). The use of biocontrollers can help to overcome these problems. The reduction of insects, parasites, and/or pathogens to a desired level using natural enemies is called biocontroller. The term biocontroller also includes some pathogens that stimulate the growth of the plant (Radtke 1993).

9.4.1 Plant Growth-Promoting Rhizobacteria

One of the most commonly used biocontrollers in crops is rhizobacteria, which promotes plant growth by stimulating systematic resistance in the host system against a diverse range of phytopathogens as well as resistance to abiotic stress, in addition to facilitating the availability of minerals and improving the absorption of nutrients (Hariprasad et al. 2014; Heidari and Golpayegani 2012; Laslo et al. 2012). PGPR (Plant Growth-Promoting Rhizobacteria) include a number of bacterial genera such as *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacteria*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Arora 2015; Goswami et al. 2016). These beneficial bacteria can act on plant growth through direct and indirect mechanisms (Fig. 9.1).

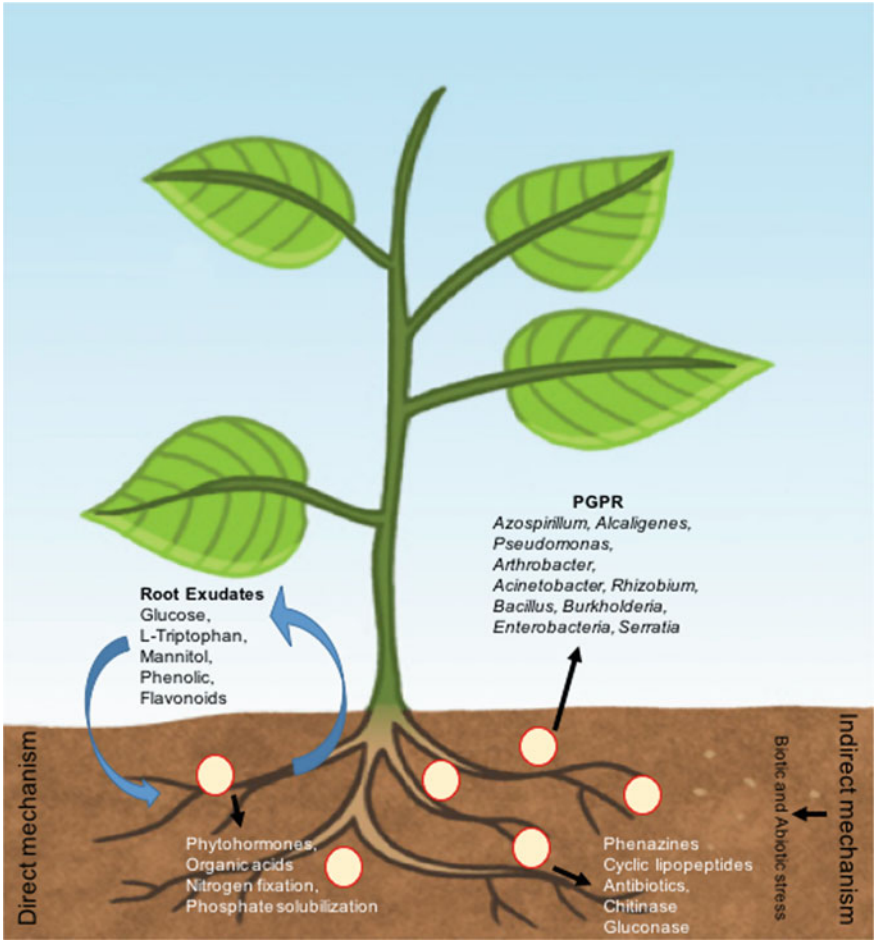

Fig. 9.1 Mechanism of plant–bacteria interaction

9.4.2 Mechanisms of Plant Promoters-Growth

9.4.2.1 Indirect Mechanisms

Indirect mechanisms for the suppression of phytopathogens include the production of siderophores, hydrogen cyanide (HCN), phenazines (PHZ), phloroglucinol, pyoluteorin (PLT), ammonia, pyrrolnitrin (PRN), antibiotics, volatile metabolites, cyclic lipopeptides (CLPs), etc., and provide protection against unhealthy environment conditions. These compounds are SMs that promote plant growth through synthesis to inhibit or control or suppress pathogens; these are not the compounds that are synthesized in the plant in association with PGPR (Fig. 9.1).

9.4.2.2 Direct Mechanisms

The direct mechanisms have been linked to the production of plant hormones such as auxins, gibberellins, ethylene, abscisic acid, jasmonic acid, salicylic acid, and cytokinins and solubilization of inorganic phosphorus and nitrogen fixation (Goswami et al. 2016). The cellular processes in plants such as the response to abiotic stress and the plant–pathogen interaction are coordinated by phytohormones which act as messengers that travel throughout the plant generating an integrated response. In addition to the above, the roots directly react to the environmental conditions through secretion of various compounds as a result of plant–bacteria interaction, which leads to suggest that this interaction is a determining factor in the efficacy of the inoculants (Cai et al. 2012; Carvalhais et al. 2013) (Fig. 9.1). The roots secrete 10–44% of the photosynthates as root exudates, which are used by microorganisms through their biochemical pathways to get energy, in addition to serve them as signaling molecules and even as antimicrobials to control microbes (Guttman et al. 2014). In addition to the above, roots also secrete ions, free oxygen and water, enzymes, mucilages, and a great variety of primary and secondary metabolites (Bais et al. 2006). For example, members of the *Poaceae* family in their early stages synthesize SMs such as benzoxazinoids (BXs) that help in the defense of the plant and allelopathy (Neal et al. 2012). However, the synthesis of these compounds (BXs) in maize depends on the amount and type of inoculation used when using different *Azospirillum* strains (Walker et al. 2011, 2012). Inoculation of different strains of *Azospirillum* (4B and B510) in rice cultivars (Nipponbare and Cigalon) showed that the type of inoculation influences the profiles of the secreted SMs, and it was observed that the phenolic compounds, flavonoids, and hydroxycinnamic derivatives showed differences between cultivars and type of strain used (Chamam et al. 2013). The inoculation of *Pseudomonas putida* strain AKMP7 in plants stressed by heat induced an improvement on the levels of metabolites such as proline, chlorophyll, sugars, starch, amino acids, and proteins and reduction in membrane damage and endogenous antioxidants enzymes such as SOD, APX, and CAT, activities compared to non-inoculated plants exposed to heat stress (Shaik et al. 2011).

The exposure of plants to PGPR protects them from stresses and pathogens. PGPR controls the bacterial population within plant tissues through modifications in the synthesis of metabolites linked to the fine control of bacterial populations (Chamam et al. 2013; Straub et al. 2013). The presence of PGPR not only influences the concentration of phytohormones, but also influences the expression of genes (Camilios-Neto et al. 2014). Colonization of *Azospirillum brasilense* in wheat induced changes in the expression of 776 ESTs of various categories such as transport, defense mechanism, and production of phytohormones (Camilios-Neto et al. 2014). This aspect has been studied the most in addition to nitrogen and phosphorus fixation. However, there are no studies on mechanisms how the plant–bacteria symbiosis influences the production of SMs in plants inoculated with PGPR.

9.5 Secondary Metabolites and Its Production/Inhibition in Plants by Nanoparticles Use

9.5.1 Nanotechnology and Nanoparticles

Nanotechnology is a new branch of science that is changing the market around the world. A physicist Richard Feynman presented the idea of nanotechnology in his talk entitled "there's plenty room at the bottom" at an American Physical Society meeting. Nanotechnology is defined as "the development, synthesis, characterization, and application of materials and devices by tailoring their shape and size at the nanoscale" (Feynman 1960; Jena and Raj 2007; Jin et al. 2001; Roy et al. 2018). Nanotechnology has been used in sciences like physics, chemistry, biology, materials science, pharmaceutical, medicine, and agriculture (Duhan et al. 2017). The particles that play an important role in nanotechnology are defined as small objects that have the same behavior as an entire unit with the same properties (Arruda et al. 2015). The particle's diameter is an important factor in their classification, coarse particles are those that cover the range of 10,000–2500 nm, fine particles are between 2500 and 100 nm, and nanoparticles are those sized between 1 and 100 nm (Arruda et al. 2015; Ghosh and Pal 2007).

9.5.2 Morphology and Shapes of Nanoparticles

Scientists have focused on intimate relationships among valence, stoichiometry, molecular geometry, and reactivity of the nanoparticles. The morphology and shape are crucial factors for the properties of the nanoparticles (NPs). The most important factor is the surface energy in order to obtain the desired shape (Sau and Rogach 2010). An important criterion for the design of new nanoparticles is the geometry obtained. In order to get different NPs shapes, it is important to design synthesis routes to obtain materials in 1D, 2D or 3D, in order to increase the catalytic activity (Matijevic 1981; Subhramannia and Pillai 2008). In conclusion, controlling the morphology is the essential requirement for the applications of the nanoparticles. They exist in different shapes such as nanocubes (Habas et al. 2007; Im et al. 2005), nanorods (Grzelczak et al. 2006; Sau and Murphy 2004), nanowires (Hunyadi and Murphy 2006; Zhao et al. 2004), prisms (Jena and Raj 2007; Jin et al. 2001), and pyramids (Burgin et al. 2008; Maiti et al. 2015). In order to classify the morphology of nanoparticles, three families can be established according to their growth: (i) 1D nanoparticles, (ii) 2D nanoparticles, and (iii) 3D nanoparticles. 3D nanoparticles are those with the main growth in three dimensions such as cubes, octahedral, pyramids. For these particles, the growth is under surface-control conditions and can be determined by crystalline structure of the particle facets. If a deck structures, the growing crystal is limited by 1 0 0 and 1 1 1 facets. In particular conditions, the 1 1 1 facets grow slower than 1 0 0, and the final shape is an octahedron limited by slow grow-

ing of 1 1 1 facets (Kim et al. 2004; Wang 2000). 2D nanoparticles have a variety of planar structures such as nanoprisms, nanoplates, nanodisks, and nanobelts. The main control of shape in this particle is the synthesis method. For example, in reduction methods, the surfactant–crystal–plane interaction energy explains the shapes as nanodisks, triangular nanoplates and nanospheres (Jiang et al. 2007; Qinghua et al. 2007). 1D nanoparticles, such as nanorods, nanowires, and nanobipyramids, are obtained by colloid chemical methods. Factors that can affect the morphology are the pH, nature of heat treatment, etc. (Murphy et al. 2005).

9.5.3 Synthesis Methods

A number of approaches have been used for the synthesis of nanoparticles; the most widely used are gas condensation (Khodaei et al. 2016; Ock et al. 2018), ionized cluster beam deposition (Araghi et al. 2016; Verrelli and Tsoukalas 2014), chemical vapor deposition (Ciprian et al. 2018; Ni et al. 2018), pulse laser deposition (Ghidelli et al. 2018; Gontad et al. 2017), thermal evaporation (Gao et al. 2016; Lachebi et al. 2018), electrochemical deposition methods (Jang et al. 2017; Sun et al. 2018), and sol–gel techniques (Gao et al. 2018; Mora et al. 2018). The emphases of the selected methods are on the control over the cluster size, shape, and dispersion on the support, and the reproducibility and low cost. The main prerequisites are preserving the morphology and the distribution of the particle size. Another consideration is on the pre-treatment process that does not affect the morphology and shape of the material.

9.5.3.1 Characterization Methods

The characterization methods that are employed are important in order to have a better understanding of nanoparticle's (NPs) properties and applications. A large number of techniques are employed in order to know the composition, morphology, coating, size, etc. (Hassellöv et al. 2008). The most used characterization techniques are:

- UV–Vis spectroscopy provides information of the particle aggregation and the average of particle size.
- Near infrared (NIR) and Fourier-transform infrared spectrometry (FTIR) identify functional groups of organic compounds that coat the nanoparticle surface.
- Inductively coupled plasma mass spectrometry or field-flow fractionation inductively coupled plasma mass spectrometry quantifies the size and distribution of the nanoparticles.
- X-ray diffraction (XRD) displays information about the crystalline state and the constitution and size of the nanoparticles.
- Dispersive spectroscopy X-ray (EDX) is used to gain elementary semiquantitative information about the material.

- High-performance liquid chromatography (HPLC) gives information of nanoparticles around 10 nm, using separation techniques.
- Atomic force microscopy (AFM) characterizes the atomic topography in which the sides are overestimated.
- Scanning electron microscopy (SEM) is applied to evaluate the size, shape, and particle aggregation.
- Transmission electron microscopy (TEM) allows the visualization of organic materials and also evaluates the size, shape, and particle aggregation.

In order to choose the better characterization technique, one should consider the complexity of the material, matrix, and the concentration of the obtained nanoparticles.

9.5.4 Secondary Metabolites

Plants produce a wide variety of low-molecular weight compounds. Among these large numbers of compounds, only a few of them are part of the primary metabolic pathways. SMs (Secondary Metabolites) are defined as organic compounds that are not directly involved in growth, development, and reproduction, but play a great role in plant adaptation and defense. These compounds are restricted to a selected plant groups (Pichersky and Gang 2000).

Plant metabolism is divided majorly into two classes. Primary metabolism in plants is the one which allows the plant to utilize water, CO_2, and minerals to synthesize the essential primary metabolites (sugars, fatty acids, amino acids, and nucleic acids) required to make and maintain the cells. The natural products or specialized metabolites are also referred as secondary metabolites and are considered to be those chemicals that the plants produce during their interactions with environment, to protect themselves from pest and pathogen and ultraviolet B radiation. The interaction of plants with the biotic environment creates the perfect scenario to produce new secondary metabolites, and as such, the majority of secondary metabolites are lineage-specific (Kliebenstein and Osbourn 2012).

Most of the secondary metabolite classes produced by plants can be classified into three main groups: (i) phenolic compounds, (ii) terpenoids/isoprenoids, and (iii) nitrogen- or sulfur-containing compounds (Fig. 9.2). As shown in Fig. 9.2, the three major classes of these metabolites are produced by different primary metabolites pathways, including glycolysis, the TCA cycle, aliphatic amino acids, pentose phosphate pathway, shikimate pathway, and notably the aromatic amino acids (Aharoni and Galili 2011).

On the other hand, plant secondary metabolites are invaluable resources, useful as food additives, fragrances, pigments, and medicines. Typically, more than 5×10^5 different flavonoids have been identified and many of them have become important nutraceuticals and pharmaceuticals (Zhang et al. 2011).

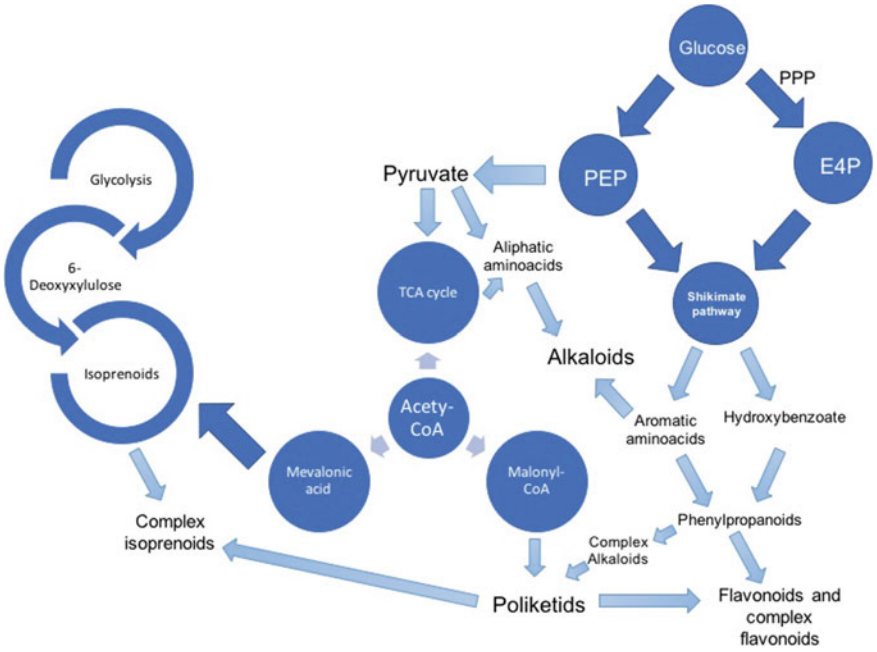

Fig. 9.2 Secondary metabolites cycles

It has been noticed in several research papers that most of the plant secondary metabolites have some beneficial roles in the human body. The SMs compounds are also known as natural products or phytochemicals, which are responsible for the medicinal properties of plants. Classification of secondary metabolites is based on the chemical structure, composition, solubility in various solvents, or their biosynthetic pathway (Misra et al. 2016).

Metabolites are small bioactive molecules that have diverse valuable properties such as pharmaceuticals, nutraceuticals, and agrochemicals. The extracts of specific plants contain some SMs/novel bioactive constituents such as phenolics, flavonoids, alkaloids, resins, quinones, steroids, and terpenoids, which are responsible for the reduction of ionic compounds to bulk metallic NPs (Aromal et al. 2012).

SMs are generally present at low concentrations in many medicinal plants. In search of alternatives to increase the production of desirable medicinal compounds in plants or SMs, nowadays the use of nanotechnology approach, specifically nanoparticles, is found to have potential as a supplement to traditional horticulture (Misra et al. 2016). NPs present unique physicochemical properties, and some studies have shown that they have the potential to boost the plant metabolism (Giraldo et al. 2014).

Only a few studies have reported the enhancement of SMs with NPs treatments under in vivo condition, and the different effects of the use of different NPs specially on plant growth and metabolic function have been observed (Nair et al. 2010). Selection of appropriate concentration, morphology, and type of nanoparticle is essential

for higher benefits of a target agroeconomic trait. Also, the enhancement in SMs can be obtained under in vitro conditions, where NPs are used as "elicitors," which are presented in Sect. 9.2.1.

9.5.5 Metallic Nanoparticles and Their Interaction with Secondary Metabolites

Plants are the vital component in biotic and abiotic systems. Their metabolism is based on events in enzyme-catalyzed reactions. The metallic nanoparticles can have positive, negative, and neutral effects. The most used metallic nanoparticles are silver, copper, and iron.

9.5.5.1 Ag Nanoparticles

Silver nanoparticles are used as antiseptic and in the process for water purification (Durán et al. 2007). Song et al. (2013) showed phytotoxicity by lower chlorophyll production, less fruit productivity, and higher superoxide dismutase activity (Song et al. 2013). An increase in levels of reactive oxygen species, superoxide dismutase, peroxidase and catalase activity, antioxidant glutathione, and malondialdehyde content was observed with silver nanoparticles (6 nm) in *Spirodela polyrhiza* (Jiang et al. 2007). Mehrian et al. (2015) studied the effects of silver nanoparticles on the contents of free amino acids and protein, lipid peroxidation and antioxidant enzymes activity, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) in tomato plants. Silver nanoparticles with 20 nm were used at five different concentrations, 0, 25, 50, 75, and 100 mg L^{-1}. The greater increase in amino acid content was observed with 75 and 100 mg L^{-1} concentrations. Tomato plants showed increase in glutamine and asparagine concentrations. The activities of SOD, CAT, and POX were increased in shoots as well as in roots of the treated tomato plants. These results highlight the modulation of oxidative stress induced by AgNPs in tomato plants (Mehrian et al. 2015). Silver nanoparticle exposition can also result in alterations in the expression of genes that are involved in formation of heat shock proteins in cells.

9.5.5.2 Cu Nanoparticles

Copper nanoparticles are used in coating on textiles for the antifungal properties. Song et al. (2015) studied the exposure of *Spirodela polyrhiza*, *Lemna minor*, and *Wolffia arrhizal* to copper nanoparticles (25 nm). The copper ions contributed to the inhibiting effects of copper nanoparticles. The Cu nanoparticles showed inhibitory effect on the total frond area-based relative growth rate, suggesting different physiological processes involved on the exposure to nanoparticles and copper ions (Song

et al. 2015). Xiao et al. (2016) studied the toxicity of Cu nanoparticles to *Daphnia magna* changed upon modification of the exposure conditions. The dissolved organic carbon (DOC) is the effective concentration of toxicity due to Cu nanoparticles obtained from laboratory tests which may overestimate the risk of the particles in polluted waters due to the common absence of DOC in laboratory test solutions (Xiao et al. 2016).

9.5.5.3 Fe Nanoparticles

The iron nanoparticles are applied for remediation of inorganic and organic contaminates in water and groundwater. The high reactivity of nanozerovalent iron (nZVI) in combination with their high specific area is very effective in remediation than granular ZVI. However, the iron can be bio-accumulated in plants. Libralato et al. (2016) studied the effects of ionic ($FeCl_3$), micro-and nano-sized zero valent iron on *Lepidium sativum*, *Sinapis alba*, and *Sorghum saccharatum*. They observed moderate biostimulation effects at the highest exposure concentrations due to potential uptake phenomena and macroscopically black spots and coatings detected on roots of all species. However, in long term exposure assays limiting the bioavailability and stressing the organisms of study were elucidated (Libralato et al. 2016).

9.5.5.4 Oxide Nanoparticles

Engineered nanomaterials (NMs) manufactured by different techniques are grouped into four types: (i) carbon-based, (ii) metal-based, (iii) metal oxides, and (iv) dendrimers and composites (Ju-Nam and Lead 2008). Among the diverse NMs, the NPs derived from diverse oxides have been used to study their roles in protection of plants against several abiotic stresses. The size and surface area of the NPs provide access for toxic metals for binding and thus reduced availability and toxicity of metals. On the other hand, the photosynthesis is a highly vulnerable cellular process. Nevertheless, NMs have shown protective ability for the photosynthetic system and improved photosynthesis by suppressing oxidative and osmotic stresses. Besides from their beneficial effects, several NMs show toxicity symptoms for plants (Feregrino-Perez et al. 2018).

9.5.5.5 SiO_2

One of the most studied oxide NPs is the silica (SiO_2), which are used directly in plant growth and development and in alleviation of abiotic stress (Feregrino-Perez et al. 2018), but they are excellent carriers of nutrients and/or pesticides; however, their effect has not been reported on the secondary metabolite production or inhibition. Nevertheless, the combination of metal and SiO_2 NPs showed the potential of silver nanoparticles as novel and effective material to modify the production of SMs inside

the plant (Moreau et al. 2013). Another example for this kind of NPs is the exposure of Ag-SiO_2 core–shell nanoparticles to *Artemisia annua* has resulted in increased artemisinin content in the hairy root culture (Ben et al. 2013).

9.5.5.6 TiO_2

The effect of TiO_2 NPs on a medicinal plant *Salvia officinalis* was studied by spraying the leaves with various TiO_2 NP concentrations (0, 10, 50, 100, 200, and 1000 mg L^{-1}). TiO_2 NPs significantly improved the total leaf phenolics and flavonoids contents compared to the control, and but no significant differences were observed in extract yield among all the treatments (Ghorbanpour 2015). This may be due to the extraction solvent type (methanol, ethanol, acetone, and water) and extraction time (Shimada et al. 1992). It has been reported that the leaf extract (methanol 90%) of *Salvia officinalis* plant exposed to TiO_2 NPs at 200 mg L^{-1} showed strong antioxidant activity compared to the control (Ghorbanpour 2015). A synergistic effect exists between phenolic and flavonoid compounds for antioxidant activity. Therefore, phenolic compounds protect the plants against oxidative damage by reducing ROS toxicity on cellular components (Hatami et al. 2016). Enhanced essential oil content (%) and yield (g/plant) of *S. officinalis* plants were reported on exposure to all concentrations of TiO_2 NPs. At moderate concentration (200 mg L^{-1}), TiO_2 NPs caused the highest essential oil content and yield which were 1.75 and 2.74 folds higher than the control plants, respectively. In another study, TiO_2 NPs sprayed within 10–15 nm size and 20–80 mg L^{-1} concentration on *Hyoscyamus niger* plants improved the tropane alkaloid (hyoscyamine and scopolamine) content and the total alkaloid yield (Hatami et al. 2016). GCMS-based metabolomic approach was used to study the toxicity of TiO_2 NPs on hydroponically cultured rice (*Oryza sativa*) plants after exposing them to 0, 100, 250, or 500 mg L^{-1} of NPs concentration for 14 days (Wu et al. 2017). Results showed that the biomass of rice was decreased due to the interference in antioxidant defense system.

One hundred and five metabolites were identified, and most of them were significantly different compared to the control. Among these metabolites, the concentrations of glucose-6-phosphate, glucose-1-phosphate, and succinic and isocitric acid were increased, while those of sucrose, isomaltulose, and glyoxylic acid were decreased. But, the biosynthesis of most of the identified fatty acids, amino acids, and SMs correlated with rice quality was increased.

9.5.5.7 ZnO

The phytochemical characteristics of *Stevia rebaudiana* under ZnO NPs concentration (100 mg L^{-1}) revealed significant increase in total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (Javed et al. 2018b). The stress to plants increased by increasing the concentration of ZnO due to the for-

mation of reactive oxygen species (ROS). Also, after reaching a certain threshold, different physiological parameters of callus and production of antioxidants decreased (Javed et al. 2017, 2018a). Soybean grown in soil previously treated with ZnO NPs maintained growth, yield, and N_2 fixation potential similarly to the controls, without increase in leaf ROS or lipid peroxidation. Leaf damage was observed by the ZnO NPs treatments (0.05, 0.1, or 0.5 g kg^{-1} soil), and genotoxicity appeared at the highest concentration, but only for one plant. Total chlorophyll decreased with increase in leaf Zn concentration, which was attributable to zinc complexes in the leaves (Priester et al. 2017). Exposure of *Glycyrrhiza glabra* seedlings grown in vitro containing Hoagland nutrient solution to 1 and 10 μM ZnO NPs enhanced the phenolic compounds and glycyrrhizin (natural sweetener) content compared to bulk ZnO (Oloumi et al. 2015). Cluster bean's 14-day-old plants were sprayed with 10 mg L^{-1} of ZnO NPs showed an improvement in the gum content and an increase in its viscosity. These results suggest that the change in these parameters might be due to the NP adsorption on plant surface and their uptake by the plants through natural microscale openings and pores (Raliya and Tarafdar 2013).

9.5.5.8 CeO_2

Soybean leaves treated with CeO_2 NPs showed increase in ROS, lipid peroxidation, and visible damage along with decrease in the total chlorophyll content. These effects were not only on aboveground plant parts, leaf, pod, and stem but also on root nodule N_2 fixation (Priester et al. 2017). In *Raphanus sativus*, no effect of 250 mg kg^{-1} of CeO_2 NPs (8 nm size) was detected on phenolic compounds, flavonoids, and nutrient accumulation in adult plants; however, antioxidant capacity of tubers was increased (Zhao et al. 2016).

9.5.5.9 CuO

Copper oxide NPs have also been included in the list of NPs used to improve the SM production. CuO NPs at 10 mg L^{-1} concentration increased the phenolic compounds and DPPH free radical scavenging activity (Javed et al. 2018b). These nanoparticles are toxic to *Stevia callus* and open opportunities for further studies for the enhancement of commercially important SMs in different medicinal plants (Javed et al. 2018b). Chinese cabbage is an important vegetable and rich source of phytochemicals such as glucosinolates (GSLs) and phenolic compounds (PCs) that are used for pharmaceutical industries. Influence of copper oxide nanoparticles (CuO NPs) on the phytochemicals (GSLs and PCs) and their biological (antioxidant, antimicrobial, and antiproliferative) activities as well as gene expression levels in Chinese cabbage has been studied (Chung et al. 2018). CuO NPs exposure escalated glucosinolates (gluconasturtiin, glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin, glucoallysin, glucobrassicanapin, sinigrin, progoitrin, and gluconapin) and transcript (*MYB34*, *MYB122*, *MYB28*, and *MYB29*) levels in cab-

bage. Moreover, phenolic compounds (flavonols, hydroxybenzoic, and hydroxycinnamic acids) were also significantly enhanced (Chung et al. 2018).

9.5.5.10 CdO

The pot-grown plants at two-leaf stage on exposure to CdO NPs of size 760 nm at concentration of $2.03 \pm 0.45 \times 10^5$ particles cm^{-3} in air for 3 weeks showed a significant effect on the total amino acids content and saccharides but no significant effect on the total SMs such as phenolic compounds etc. as revealed by chromatographic assays (Večeřová et al. 2016).

9.5.5.11 Al_2O_3

Use of Al_2O_3 NPs on soybean seedlings affected their growth, root rigidity, and root cell viability (Mojiri et al. 2016). Further severe oxidative burst was evident. Gel-free proteomic analysis of stressed soybean plant's roots showed 104 changes in proteins associated with SMs, cell organization, and hormone metabolism. Oxidation–reduction-related genes such as GDSL motif lipase 5, SKU5 similar 4, galactose oxidase, and quinone reductase were modified in Al_2O_3 NPs stressed roots. These proteomic changes suggested that high concentration of proteins involved in oxidation–reduction, stress signaling, and hormonal pathways related to growth and development might be the crucial key for optimum growth of soybean under Al_2O_3 NPs treatment (Hossain et al. 2016). As shown in Table 9.4, several studies were undertaken on use of SiO_2 NPs on different plant species under different abiotic stresses. Further studies are required to study their effects on the secondary metabolite production.

9.6 Metabolic Engineering Strategies

Plants have a myriad of metabolic pathways responsible for the biosynthesis of secondary and specialized metabolites with biological activities of anthropocentric interest. The omics tools have boosted the identification, isolation, and application of new genes corresponding to these metabolic pathways. Heterologous plant hosts can be reconstituted in order to overproduce these high-value compounds. As alternative, the plant biochemical pathways can be modified using molecular strategies, based on previous insights of the biochemical routes desired to be modified, to overproduce these compounds. This approach is known as "Metabolic Engineering," and the strategy highlights several examples that demonstrated the actual possibilities on how can the metabolic pathways in plants be harnessed for human welfare. Plants respond to environmental stress conditions by producing several metabolites such as phenolic compounds, alkaloids, and terpenes, in order to cope and adapt to these stresses. These

Table 9.4 Effect over diverse plants and secondary metabolites production

Material	Plant cell culture	Effect/secondary metabolites enhanced	References
SiO_2	*Crataegus* sp.	Positive effect on photosynthetic rate and no effect on chlorophyll and carotenoid content	Ashkavand et al. (2016)
	Lycopersicum esculentum	Increased seed germination at low concentrations	Haghighi et al. (2012)
	Ocimum basilicum	Increased chlorophyll and proline content	Kalteh et al. (2014)
	Lens culinaris	Enhanced seed germination and seedling growth	Sabaghnia et al. (2014), Siddiqui Manzer et al. (2014)
	Cucurbita pepo	Increased seed germination and seedling growth. Reduced chlorophyll degradation and oxidative damage, enhanced photosynthetic parameters and antioxidant enzymes	Siddiqui Manzer et al. (2014)
	Vicia faba	Enhanced seed germination and seedling growth	Qados and Moftah (2015)
	Solanum lycopersicum	RBOH1, APX2, MAPK2, ERF5	Almutairi (2016)
	Foeniculum vulgare	Benzoic acid, jasmonic acid, hexadecanoic and pyrrolidinone	Bahreini et al. (2015)
TiO_2	*Aloe vera*	Aloin	Raei et al. (2014)
	Cicer arietinum	Phenolic and flavonoid compounds	AL-oubaidi and Kasid (2015)
	Foeniculum vulgare	Dodecane, phytol, phenol 2,4 bis (1,1 dimethyl ethyl) and octane	Bahreini et al. (2015)
	Mentha piperita	Increase in nitrate reductase and carbonic anhydrase activities. Also essential oil production was enhanced	Ahmad et al. (2018a)

(continued)

Table 9.4 (continued)

Material	Plant cell culture	Effect/secondary metabolites enhanced	References
ZnO	*Hypericum perforatum*	Hypericin and hyperforin production	Sharafi et al. (2013)
	Phaseolus vulgaris	Chlorophyll, carotenoids and oxidative stress biomarkers	García-Gómez et al. (2017)
	Solanum lycopersicon	Chlorophyll, carotenoids and oxidative stress biomarkers	García-Gómez et al. (2017)

metabolites do have impact on how effectively plants can be used as functional foods. Besides, many of these plant metabolites have demonstrated benefits to human health due to their several biological activities such as antimutagenic, anti-inflammatory, and antimicrobials (García-Mier et al. 2013; Vargas-Hernandez et al. 2017; Veloz-García et al. 2010).

Despite the mentioned importance of these plant metabolites, the biosynthetic processes for only a small fraction of these complicated molecules are known, indicating that the majority of the diversity of plant metabolism has not yet explored. Recent progress in next-generation sequencing technologies, along with the development of new algorithms for bioinformatics analysis of these sequence data, has greatly boosted the process of gene discovery involved in plant metabolic processes. These discoveries have allowed advancements in engineering the plant metabolism (metabolic engineering). The unraveling of these pathways will permit us to fully harness the wealth of compounds and biocatalysts that plants provide (Tatsis and O'Connor 2016). Some recent approaches of metabolic engineering in plants are described to display the current potential of this strategy to increase high-value-based metabolites in plants.

9.6.1 Homologous Overexpression and Heterologous Reconstitution of Plant Biochemical Pathways

The possibility of reconstitution of a plant metabolic pathway in a heterologous organism is one approach well studied in metabolic engineering. For this strategy, either plants or microorganisms (mainly *Escherichia coli* and *Saccharomyces cerevisiae*) have been successfully used. In the case of plants, tobacco (*Nicotiana tabacum*) displays a quick and cheap plant model to be used for this purpose (Jiang et al. 2015). Moreover, other authors have elegantly delineated a ten-step pathway of a genetically intractable plant species to produce the etoposide aglycone in *Nicotiana benthamiana* by assembly of the entire functional pathway in this species (Lau and Sattely 2015). Pharmaceutically important terpenoids of plant origin, as those

from *Panax ginseng* (ginsenosides) have been increased by twofold in transgenic *P. ginseng* overexpressing the ginsenosides biosynthetic pathway key gene PgSQS1, that up-regulate the expression α-amyrin synthase (α-AS) and cycloartenol synthase (CAS) (Shim et al. 2010). Similar approaches using overexpression of other key genes in the ginsenosides biochemical pathways have also been successfully reported (Kim et al. 2014).

Metabolic regulation of multiple key genes in several biosynthetic pathways can effectively increase the pharmaceutical terpenoid content in medicinal plants. Tanshinones, abietane-type norditerpenoid quinones in *Salvia miltiorrhiza*, have antibacterial, anti-inflammatory, and broad antitumor activities (Gao et al. 2014). The introduction of the *SmHMGR* and/or *SmGGDS* genes as well as *SmDXS* gene in *S. miltiorrhiza* hairy root lines results in a significant enhancement of tanshinone production (Kai et al. 2011). Moreover, co-expression of the *SmHMGR* and *SmGGDS* genes resulted in the highest production of tanshinone. Overexpression of artemisinin biosynthesis genes *ADS* (Amorpha-4,11-diene synthase gene), *CYP71AV1* (cytochrome P450-dependent hydroxylase gene), and *CPR* (NADPH: cytochrome P450 oxidoreductase gene) promoted the accumulation of artemisinin in *Artemisia annua* (Lu et al. 2013). Other approaches for increasing triterpenoids in plants have been reviewed elsewhere (Moses et al. 2013). Flavonoids, a group of polyphenolic plant SMs, are important for plant biology and human nutrition. In particular, flavonols are potent antioxidants, and their dietary intake is correlated with a reduced risk of cardiovascular diseases. Tomato fruit contains only in their peel small amounts of flavonoids, mainly naringenin chalcone and flavonol rutin, a quercetin glycoside. The transgenic expression of maize transcription factors genes LC and C1 in tomato fruits significantly up-regulated the flavonoid pathway in tomato fruit flesh, a tissue that normally does not produce any flavonoids. These fruits accumulated high levels of kaempferol (a flavonoid) in their flesh (Bovy et al. 2002).

9.6.2 Gene Silencing Strategies

Another approach to modify plant metabolites is the down-regulation of specific genes involved in the competitive pathway in order to enhance the production of desired compounds. Virus-induced gene silencing (VIGS) has been used to evaluate the effect of silencing of some genes of capsaicinoid biosynthetic pathway in pepper. The silencing of *Comt*, p*Amt*, and *Kas* genes using a geminiviral vector demonstrated that capsaicin was no more produced in fruits (del Rosario Abraham-Juárez et al. 2008). The frontier tool for gene editing called clustered regularly interspaced short palindromic repeats (CRISP)/CRISPR-associated9 (Cas9) endonuclease system has recently been found useful in knocking out the gene 4′OMT2 in opium poppy (*Papaver somniferum*) to regulate the biosynthesis of benzylisoquinoline alkaloids (BIAs) (Alagoz et al. 2016).

9.6.3 Constructing Specific Molecules

Commercial DNA synthesis currently makes it possible to rapidly generate single- and multi-gene constructs for expression in the heterologous systems. In the previous years, further growth and innovation in the DNA synthesis industry has continued to diminish the price of synthetic genes to the extent that purchasing DNA is often competitive with the cost of PCR cloning, particularly when subsequent sequence manipulation is required to obtain the exact sequence desired for experimentation (Owen et al. 2017). In this scenario, it is expected in short time to design specific gene constructs in order to rapidly provide the possibility of introducing a complete biosynthetic pathway or to modify it according to the desired metabolites to be produced by plants or heterologous hosts. Plants have a number of advantages as heterologous hosts for metabolic engineering. They require only simple inorganic nutrients, water, carbon dioxide, and sunlight for their efficient growth. Moreover, they are also more amenable to expression of genes of plant origin than microbes since they support correct mRNA and protein processing, protein localization. and metabolic compartmentalization, and already have many of the necessary metabolic precursors and co-enzymes (Owen et al. 2017). As non-hosts for animal and human pathogens, they are an attractive alternative to human cell cultures for the production of vaccines and therapies (Geu-Flores et al. 2012). Thus, combining the strategies of synthetic biology with metabolic engineering would be faster to produce the desired metabolites in plants.

9.6.4 Non-genetically Modifying Approach (Eustressors)

Plants induce their immune system to cope up with environmental stresses either from biotic or abiotic agents (Cardenas-Manríquez et al. 2016). Among the arsenal of defense against stresses, plants produce a myriad of specialized metabolites (much of them with biological activities of anthropocentric interest). According to the theory of stress, if this situation corresponds to a “mild stress” that flavors somehow plant performance, including bioactive production; the stress is called “eustress” or good stress (Hideg et al. 2013). On the contrary, if the stress level is high enough to provoke minor growth and even plant death, the stress is called “distress” or bad stress (Hideg et al. 2013). Thus, several approaches searching for eustress factors of biological (elicitors), chemical, or physical nature have been reported with positive results of inducing the production of specific metabolites. For instance, in the case of chili pepper, biological and chemical eustressors caused a significant increase in capsinoid levels (Zunun-Pérez et al. 2017). In addition, physical eustressors such as UV-B treatment on several crops as tomato and lettuce have also been demonstrated to increase flavonoids in several plant organs (Neugart and Schreiner 2018). Interestingly, the application of these eustress-inducing factors has been demonstrated to be inherited to the offspring using epigenetic mechanisms (Avramova 2015).

9.7 Conclusions and Future Perspective

The SMs are economically important due to their use in pharmaceutical, food, cosmetics, and agricultural industries. Current trend is to produce adequate, nutritious, and safe food (with less chemical substances such as fertilizers, herbicides, pesticides, antifungi) that not only nourish, but also contribute to an extra health benefit (nutraceutical) with minimum inputs and without greatly compromising the production of the plant and environment. Several approaches or strategies have been used to produce secondary metabolites in the plants for human health benefits. Although SMs are the result of adaptation and evolution of the plant in the face of changes in the environment, they can be induced by factors that resemble changing environmental conditions as biotic and abiotic factors. These factors act as elicitors which at an effective dose at a specific plant phenology induce the production of SMs that influence the plant growth and development and protect them against pests and pathogens. These compounds (elicitors either endogenous or found in soil, phytohormones, bacteria, fungi, and organic compounds) are marketed as biostimulants (as a mixture or in aqueous solution) and are currently used in various countries as an alternative to organophosphorus pesticides and fertilizers that can contaminate aquifers and soils. Controversy of their use lies in the difficulty to create or adapt legislations for their application in growing areas. Moreover, these products are placed in the market following national and supranational regulation of fertilizers and pesticides (du Jardin 2015). Because of their origin in nature, they offer an alternative to produce food free of harmful agents that can induce diseases.

The other aspects are the biotechnological advances, application of nanomaterials, and metabolic engineering. Nowadays, nano-biotechnology industries are growing very rapidly; however, there is an urgent need to perform profound studies in this field in order to develop comparatively safe and eco-friendly nanoparticles in the long run. The widespread assessment of these NPs in agri-food sector should also be carried out for public acceptance to prevent them from the unlike challenges as were faced by genetically modified organisms worldwide. Although there is a great controversy about the positive and negative aspects of the application of nanomaterials or nanoparticles on crops, it is clear that the impact of their application depends on the type of crop, the time of exposure, the crop developmental stage at which they are applied, as well as the concentration and type of material used. Further, the technological advances have made it possible to identify, characterize, and isolate novel genes for the manipulation of diverse plant metabolic pathways to improve SM production. However, despite generation of the vast amount of information in the last decades, it is still not possible to fully elucidate the behavior of the stressors, leaving behind several points unaddressed, as well as the various experimental models to be tested.

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Chapter 10
Development of *Brassica* Oilseed Crops with Low Antinutritional Glucosinolates and Rich in Anticancer Glucosinolates

Naveen C. Bisht and Rehna Augustine

Abstract Glucosinolates are a class of plant secondary metabolites of *Brassicaceae* family with diverse biological functions. Hydrolytic products of some glucosinolates are beneficial whereas some impart antinutritional properties. Sulforaphane, the degradation product of glucosinolate glucoraphanin is known as one of the most potent naturally occurring anticancer compound. Sulforaphane protects the body against a variety of chronic diseases. The major antinutritional effect of glucosinolates reported is its interference with thyroid function, especially in livestock and poultry which are routinely fed on rapeseed–mustard meal. For example, 2-hydroxy-3-butenyl glucosinolate forms an oxazolidine-2-thione upon hydrolysis which is goitrogenic. The presence of antinutritional glucosinolates drastically reduces its food and feed value and hence also its market value. It is therefore, imperative to develop *Brassica* oilseed crops which are rich in beneficial glucosinolates and low in antinutritional glucosinolates. Conventional breeding efforts as well as recent biotechnological advances have contributed largely toward this goal. Recently, using RNAi-mediated silencing of the *GSL-ALK* gene, high accumulation of glucoraphanin was achieved in the seeds of *Brassica juncea* with a concomitant decline in the concentrations of antinutritional glucosinolates. The chapter summarizes the health effects of glucosinolates, the genetics of beneficial glucosinolate accumulation in *Brassica* crops and the current status of research toward the enrichment of *Brassica* crops with beneficial glucosinolates.

Keywords *Brassica* · Glucosinolates · Antinutritional · Glucoraphanin · Sulforaphane · GSL-ALK · RNAi silencing

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Abbreviations

AFLP	Amplified fragment length polymorphism
ALK	Alkenase
AOP	Alkenyl hydroxyl producing
BCAT	Branched-chain aminotransferases
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR associated protein
DW	Dry weight
FMO	Flavin monooxygenase
GNA	Gluconapin
GSL	Glucosinolate
GTR	Glucosinolate transporter
MAM	Methylthioalkyl malate synthase
Met	Methionine
QTL	Quantitative trait loci
RNAi	RNA interference
TILLING	Targeting induced local lesions in genomes
Trp	Tryptophan

10.1 Introduction

Brassica foods are getting attention worldwide due to their immense nutritional potential. The presence of a unique metabolite called glucosinolate makes the *Brassica* foods attractive. Glucosinolates are simple compounds originating from amino acids through a pathway with complex regulatory mechanisms which vary with species to species and environment to environment. Glucosinolates have a huge diversity accounting to more than 130 molecules reported to date. Glucosinolates possess a broad spectrum of biological activity (Agerbirk and Olsen 2012). In nature, glucosinolates form part of the innate defense machinery of plants (Hopkins et al. 2009). Glucosinolates in the native form are inert and compartmentalized in cell vacuoles; however, tissue damage of any form exposes these compounds to a class of hydrolyzing enzyme called myriosinases which is otherwise compartmentalized in specialized cells called myriocin cells. The degradation products of glucosinolates impart various biological activities (Halkier and Gershenzen 2006) (Fig. 10.1).

Brassica products are consumed as oil, meal and as vegetables. Rapeseed-mustard (*B. napus*, *B. juncea* and *B. rapa*) is a source of oil and has a protein-rich seed meal. High glucosinolates and erucic acid in the seed meal impart health risks to poultry and livestock (Fenwick et al. 1983; Griffiths et al. 1998). Canola Council of Canada (2010) has set safe limits of erucic acid and glucosinolates in the defatted seed meal as <2% and <30 μmol g^{-1} DW, respectively. Rapeseed–mustard seed meal is a protein-rich animal feed supplement. However, the presence of high amounts of glucosino-

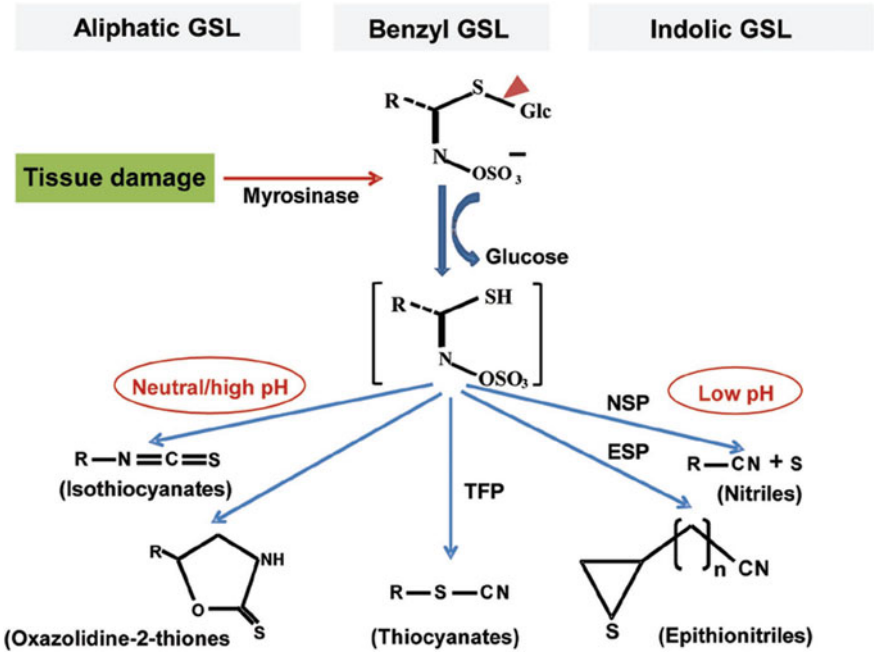

Fig. 10.1 Glucosinolates and its hydrolytic products. Upon tissue damage, glucosinolates are hydrolyzed by myrosinases. At neutral pH, the unstable aglycones rearrange to form isothiocyanates. If the glucosinolate side chain is hydroxylated at carbon 3, spontaneous cyclization of the isothiocyanate results in the formation of an oxazolidine-2-thione. In the presence of an epithiospecifier protein-like factor (ESP), nitriles are formed. Nitrile formation has been shown in some cases to be favoured at low pH (pH < 3). If there is a terminal double bond in the side chain, the sulfur atom released during nitrile formation is captured by the double bond, resulting in the formation of epithionitriles. Some glucosinolates can be hydrolyzed to thiocyanates (R is a variable side chain)

lates in rapeseed/canola seed meal makes the oil cake bitter and hence unpalatable. High contents of glucosinolates also possess serious health hazards (Augustine et al. 2013b). It is therefore imperative to genetically manipulate the glucosinolate content in *Brassica* crops so that the feed value can be greatly improved. In human diet as well, *Brassica* vegetables are highly important as they are good sources of vitamin A and C, dietary soluble fibers, folic acid, and essential micronutrients (Hirani et al. 2012). Hence, one of the main breeding objectives in *Brassica* crops is to enhance the content of beneficial glucosinolates and to reduce the deleterious glucosinolates in edible plant parts. This chapter deals with the basics of glucosinolates, their biological effects, efforts and strategies to achieve low antinutritional glucosinolates and high-glucoraphanin content in *Brassica* crops.

10.2 The History, Economic Importance and Distribution of *Brassica* Crops

Brassicaceae is one of the most important plant families of the world from the economic point of view. Brassicaceae or the cabbage family is the fifth-largest monophyletic angiosperm family of flowering plants, with 372 genera and 4060 species. The mustard family contains the widely cultivated species, such as *Brassica oleracea*, *Brassica rapa*, *Brassica napus*, *Raphanus sativus*, *Brassica juncea, Brassica nigra* and many others including the model plant, *Arabidopsis thaliana* (Rakow 2004; Johnston et al. 2005; Beilstein et al. 2006). History of rapeseed cultivation can be traced back to several thousand years with its origin in the Mediterranean region although the exact time of domestication and the place of origin are still unknown. *B. juncea* and *B. rapa* are believed to be cultivated in India long before the Christian era (Prakash and Hinata 1980). Rapeseed–mustard is the third most important oilseed in the world after soybean and groundnut. Canola oil is considered as one of the healthiest edible oils. The oil consists of high level of monounsaturated fatty acid (61%), lower level of saturated fatty acid (7%) and moderate amount of polyunsaturated fatty acid (22%) (McVetty and Scarth 2008). The oil cake left out after extraction of oil is a rich source of protein (36–39%) which is mostly used as animal feed (Cartea and Velasco 2008). In recent years, *Brassica* oil is also getting popularity in biofuel production (Hill et al. 2006).

10.3 Glucosinolate Structure, Classification and Metabolism

Glucosinolates are nitrogen- and sulfur-containing secondary metabolites with a common core structure containing a β-D-thioglucose group linked to a sulfonated aldoxime moiety and a variable side chain derived from amino acids. Glucosinolates are classified based on their amino acid precursors into three major groups: aliphatic, indolic and aromatic (Halkier and Gershenzen 2006). Aliphatic glucosinolates are derived from Ala, Leu, Ile, Val or Met and constitute the major group of glucosinolates in *Brassica* species. Commonly occurring aliphatic glucosinolates include gluconapin, sinigrin, glucoraphanin, glucobrassicanapin, glucoiberin, glucoerucin (Table 10.1). Aliphatic glucosinolates are further divided into propyl (C_3), butyl (C_4) and pentyl (C_5) glucosinolates based on the length of side chain. Indolic glucosinolates are derived from the amino acid, Trp. Major indolic glucosinolates found in cultivated *Brassica* species include glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin. Aromatic glucosinolates are derived from the aromatic amino acids, Phe and Tyr. They are less common compared to other two classes of glucosinolates. Glucobarbarin, glucotropaeolin, glucosinalbin, gluconasturtiin and glucomalcomiin are some of the commonly found aromatic

Table 10.1 Commonly occurring aliphatic (Ali), indolic (Ind) and aromatic (Aro) glucosinolates and their abbreviation

Chain length	R side chain	Trivial name
C_3 Ali	3-Methylthiopropyl (3MTP)	Glucoibervirin (IBV)
	3-Methylsulfinylpropyl (3MSOP)	Glucoiberin (IBE)
	3-Methylsulfonylpropyl	Glucocheirolin (CHE)
	2-Propenyl	Sinigrin (SIN)
	3-Hydroxypropyl (3OHP)	Glucoerysimumhieracifolium
C_4 Ali	4-Methylthiobutyl (4MTB)	Glucoerucin (ERU)
	4-Methylthio-3-butenyl	Glucoraphasatin
	4-Methylsulfinylbutyl (4MSOB)	Glucoraphanin (GRA)
	4-Methylsulfinyl-3-butenyl	Glucoraphenin (RAA)
	3-Butenyl	Gluconapin (GNA)
	(2R)-2-Hydroxy-3-butenyl	Progoitrin (PRO)
	(2S)-2-Hydroxy-3-butenyl	Epiprogoitrin (EPI)
	4-Hydroxybutyl	Glucoarabidopsisthalianain
C_5 Ali	5-Methylthiopentyl (5MTP)	Glucoberteroin (BER)
	5-Methylsulfinylpentyl (5MSOP)	Glucoalyssin (ALY)
	4-Pentenyl	Glucobrassicanapin(GBN)
	2-Hydroxy-4-pentenyl	Gluconapoleiferin (NAP)
C_6 Ali	6-Methylthiohexyl (6MTH)	Glucosquerellin
	6-Methylsulfinylhexyl (6MSOH)	Glucohesperin
C_7 Ali	7-Methylthioheptyl (7MTH)	Glucoarabishirsutain
	7-Methylsulfinylheptyl (7MSOH)	Glucoibarin
C_8 Ali	8-Methylthiooctyl (8MTO)	Glucoarabishirsuin
	8-Methylsulfinyloctyl (8MSOO)	Glucohirsutin
Ind	Indol-3-ylmethyl (I3M)	Glucobrassicin (GBC)
	4-Hydroxyindol-3-ylmethyl (4-OH)	4-Hydroxyglucobrassicin (4-OH)
	1-Methoxyindol-3-ylmethyl (1MOI3M)	Neoglucobrassicin (NEO)
	4-Methoxyindol-3-ylmethyl (4MOI3M)	4-Methoxyglucobrassicin (4ME)
Aro	Benzyl	Glucotropaeolin (GTL)
	2-Phenylethyl (2PE)	Gluconasturtiin (NAS)
	p-Hydroxybenzyl	Glucosinalbin/Sinalbin
	3-Benzoyloxypropyl (3BzOP)	Glucomalcomiin
	4-Benzoyloxybutyl (4BzOB)	–

glucosinolates (Grubb and Abel 2006; Sonderby et al. 2010; Augustine and Bisht 2017).

Biosynthesis of glucosinolates occurs from amino acid precursors through three distinct phases; side-chain elongation of selected precursor amino acid, core glucosinolates structure formation and the secondary modifications of the side chain (Fig. 10.2). The secondary modifications of the side chain lead to the structural diversity of glucosinolates. During this phase, the side chain undergoes various modification(s) such as oxidation, alkylation and/or esterification reactions. Side-chain modifications of glucosinolates occur through stepwise oxidation of methylthioalkyl moieties to methylsulfinylalkyl and then to alkenyl moieties (Field et al. 2004; Halkier and Gershenzen 2006; Sonderby et al. 2010). The flavin monooxygenase ($FMO_{GS\text{-}OX1\text{-}5}$) catalyzes the conversion of methylthioalkyl glucosinolates into methylsulfinylalkyl glucosinolates (Hansen et al. 2007; Li et al. 2008). AOP2 and AOP3, the two α-ketoglutarate-dependent dioxygenases control the conversion of methylsulfinylalkyl to alkenyl- and hydroxyalkyl glucosinolates, respectively (Kliebenstein et al. 2001a, b). Glucosinolates are predominantly synthesized in green tissues (source) and are then transported to developing seeds (sink) via long-distance phloem transport system (Chen et al. 2001; Brown et al. 2003). Nour-Eldin et al. (2012) have identified two members of the nitrate/peptide transporter family in model plant *A. thaliana*, *GTR1* and *GTR2*, as proton-dependent glucosinolate-specific transporters which are essential for long-distance transport of glucosinolates.

In *Brassica* crops, both glucosinolates content and profile are highly variable and species-specific, with aliphatic glucosinolates (derived from methionine) being the predominant glucosinolates (up to 95% of the total glucosinolates). The most common aliphatic glucosinolates in *B. rapa* and *B. nigra* are 3-butenyl and 2-propenyl, respectively. The aliphatic glucosinolates pool in *B. napus* usually contains 3-butenyl, 4-pentenyls and their hydroxyl forms. However, the *B. juncea* cultivars consist mainly of 3-butenyl and 2-propenyl glucosinolates. Furthermore, within *B. juncea*, the two heterotic gene pools are also contrasting for their glucosinolates profile and content, with east European *B. juncea* cultivars predominantly containing 2-propenyls (approx. 99%) whereas Indian cultivars primarily contain 3-butenyls with little of 2-propenyls and 4-pentenyls glucosinolates (Love et al. 1990; Pradhan et al. 1993; Sodhi et al. 2002).

Intact glucosinolates are known to be inactive compounds. However, up on tissue disruption, glucosinolates come in contact with a hydrolyzing enzyme, myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) and are converted to a variety of biologically active compounds (Fig. 10.1). The myriosinases catalyze hydrolysis of the thioglucoside linkage, leading to the formation of glucose and an unstable aglycone moiety (Halkier and Gershenzen 2006) which rearranges to form isothiocyanates, simple nitriles, epithionitriles or thiocyanates, depending on the biochemical properties of the specifier protein and the structure of the glucosinolates side chain, pH and presence of additional proteins or cofactors (Wittstock and Burow 2010; Kissen and Bones 2009). These degradation products are actually responsible for the observed biological activities of glucosinolates.

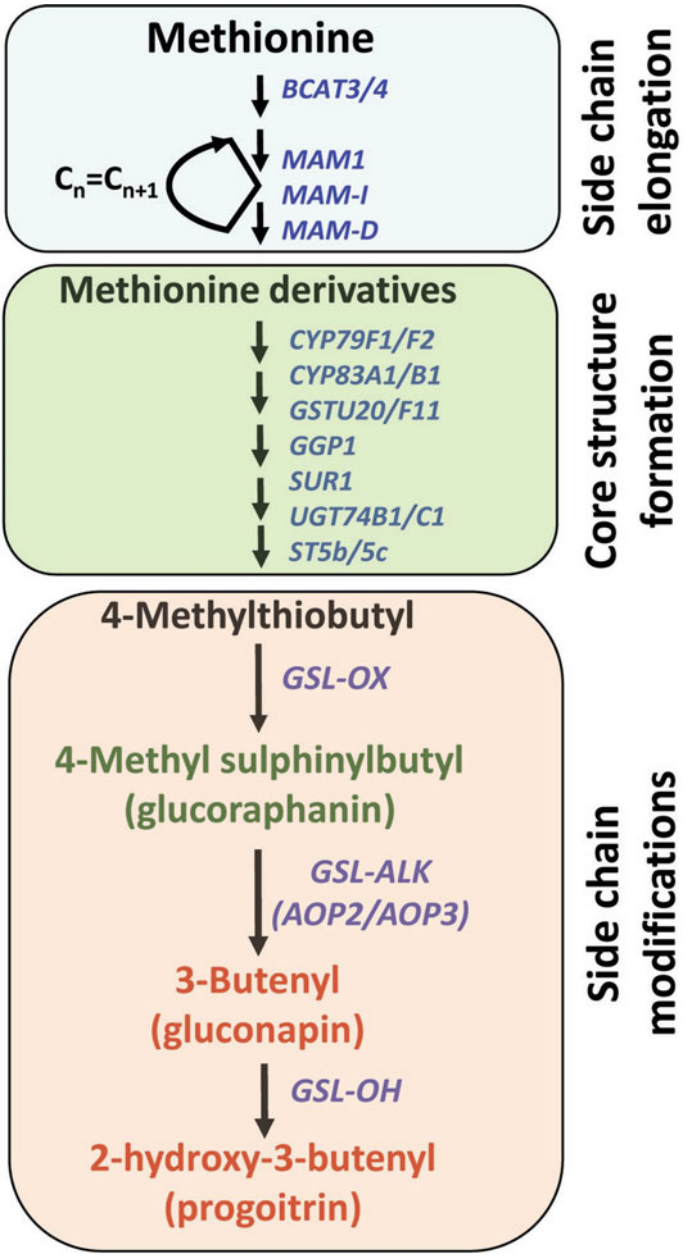

Fig. 10.2 Generalized scheme of aliphatic glucosinolate biosynthesis pathway. The biosynthesis of aliphatic glucosinolates involves three phases, namely side-chain elongation, the formation of glucosinolate core and side-chain modification reactions. The steps and genes involved in Met-derived C_4 glucosinolate reactions are shown

10.4 Biological Activities of Glucosinolate Degradation Products

Glucosinolates research has received much attention in recent years as their breakdown products are associated with several potent bioactivities. Sulforaphane, the isothiocyanate derivative of 4-methylsulfinylbutyl glucosinolate (4-methylsulfinylalkyl glucosinolate/4-MSOB), predominantly found in broccoli sprouts, has been shown to have potent anticarcinogenic properties. Sulforaphane is considered as the most beneficial glucosinolate investigated so far. Healing properties of sulforaphane, in breast, cervical, prostate, colon, stomach and UV light-mediated skin cancers have been well documented (Dinkova-Kostova et al. 2006). It is shown that sulforaphane can induce phase II detoxification enzymes, such as quinone reductase, glutathione-*S*-transferase and glucuronosyl transferases through activation of NrF2 (NF-E2 related factor 2) and AhR (aryl hydrocarbon receptor) in tumor cells (Hayes et al. 2008). Recently, a novel suppression mechanism showing the ability of sulforaphane to inhibit histone deacetylase (HDAC) enzymes, to alter histone acetylation and affect gene regulation has been reported (Myzak et al. 2007; Ho et al. 2009). Degradation product of indole glucosinolate, Indole-3-carbinol derived from glucobrassicin has reported to have potent anticarcinogenic activity (Hrncirik et al. 2001). Sulforaphane also has the potential to prevent tumor growth by blocking the cell cycle and promoting apoptosis (Bonnesen et al. 2001). Sulforaphane is also shown to be a dose-related inhibitor of carcinogen-induced mammary tumorigenesis in rats. It helps in eradicating *Helicobacter pylori* and thus prevents the incidence of gastritis and stomach cancer in mice (Fahey et al. 2001). Sulforaphane can also fight against cystic fibrosis, aging, rhinitis, arthritis, asthma and other lung disorders. Hence, regular consumption of cruciferous vegetables is highly beneficial (Fahey et al. 1997; Shapiro et al. 2001; Yanaka et al. 2009; Clarke 2010; Fahey et al. 2012).

Among the cruciferous vegetables, broccoli seeds and sprouts (3 days old seedlings) contain the highest amounts of glucoraphanin (Fahey et al. 1997; Farnham et al. 2005). Other members of *Brassica oleracea* like Chinese kale, cabbage and brussel sprout also possess significant amounts of glucoraphanin. However, most *Brassica* cultivars grown for vegetable or oil purpose contain very less or negligible amounts of glucoraphanin. Even though cultivated mainly as an oilseed, leaves of the young plants of *B. juncea* and *B. napus* are consumed as a vegetable as well. 3-butenyl (C_4), 2-propenyl (C_3) and 4-pentenyl (C_5) glucosinolates are the major aliphatic glucosinolates present in *B. juncea* (Sodhi et al. 2002). The presence of high amounts of these glucosinolates in *B. juncea* is antinutritional in nature (Augustine et al. 2013a). Some glucosinolates present in rapeseed meal fed to poultry and ruminants are found to be detrimental (Mawson et al. 1993). Its hydrolyzed products, isothiocyanates and other sulfur-containing compounds, were shown to interfere with the uptake of iodine by the thyroid gland. For example, the glucosinolate progoitrin is hydrolyzed to goitrin, or l-5-vinyl-2-thioöxazolidine, which possesses antithyroid effects. Goitrin blocks tyrosine iodination and inhibits T4 formation (Bischoff 2016). Long-term ingestion is associated with goiter formation. Progoitrin is found in rapeseed, kale and a

variety of vegetables and seeds. Likewise, the predominant rapeseed glucosinolates, 2-hydroxy-3-butenyl glucosinolate, forms an oxazolidine-2-thione upon hydrolysis which causes goiter in animals by inhibiting thyroxin synthesis which is independent of iodine availability (Mithen et al. 2000). Thiocyanate ions formed from aliphatic glucosinolates that contain a hydroxyl side chain interfere with iodine uptake in pigs and rats which can be reversed with iodine supply (Schöne et al. 1997). Although there is no clear evidence to fully describe the mechanism involved in glucosinolates-related effects on animal reproduction, lowered fertility was observed in animals fed with rapeseed meal rich in glucosinolates content (Mawson et al. 1993). Tripathi et al. (2003) have shown that high glucosinolates content (58 μmol g^{-1} DW) in the seed meal of *B. juncea* can impair food intake of rabbits. Stunted growth of poultry was also observed when fed with a glucosinolates-rich diet for a longer time (Bell 1993). However, there is not much evidence of its deleterious effects on human beings (Clarke 2010). Hence, metabolic engineering of *B. juncea* for the enrichment of desirable glucosinolate (e.g., glucoraphanin) and reducing the antinutritional glucosinolates seems highly essential to improve the food and feed value of this crop (Fig. 10.3).

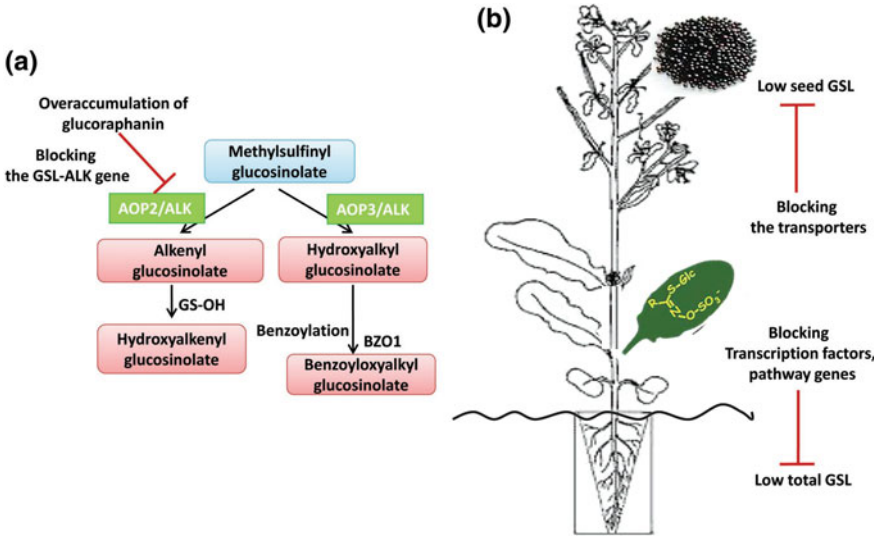

Fig. 10.3 Biotechnological strategies used for manipulation of glucoinsolate pathway in *Brassica* species. **a** Overaccumulation of desirable glucosinolate, glucoraphanin by blocking the functional *GSL-ALK* gene. **b** Targeted and tissue-specific manipulation for developing low glucosinolate lines has been attained by silencing the transcription factors (e.g., MYB28), pathway genes (MAM1) and glucosinolate-specific transporters (GTRs) of *Brassica* species

10.5 Development of Low Glucosinolate *Brassicas*

Since some glucosinolates possess deleterious effect on cattle and poultry, efforts have been made to develop low glucosinolate *Brassica* crops. The discovery of low aliphatic glucosinolates trait in the *B. napus* cultivar Bronowski (~12 μmol g^{-1} oil-free meal and 7–10% erucic acid) in Poland was a breakthrough in breeding for low glucosinolates traits in *Brassica* crops (Kondra and Stefannson 1970; Krzymanski 1970). Till now, this is the only natural source for low glucosinolate trait in *Brassica* breeding programs (Howell et al. 2003). The first low glucosinolate summer rape, cv. Tower, was registered in 1974 (Stefansson and Kondra 1975). Breeding for low glucosinolate trait in *B. juncea* started in Canada by interspecific crosses between a *B. juncea* cultivar with non-allyl, 3-butenyl glucosinolate and a low glucosinolate *B. rapa* followed by backcrossing and subsequent inbreeding (Love et al. 1990). The performance of these plants was later improved by further backcrossing with high yielding *B. juncea* line (Love et al. 1991; Rakow et al. 1995). The first canola quality *B. juncea* varieties were registered in Canada in 2002 by the Saskatchewan Wheat Pool (SWP). However, the germplasm pool of canola quality *B. juncea* is genetically quite narrow (Burton et al. 2004). A recurrent selection and backcross (RSB) method in *B. juncea* using double haploids (DH) in each generation was shown to introgress the low glucosinolate alleles from a low glucosinolate east European *B. juncea* line, Heera into an Indian variety, Varuna (Ramchiary et al. 2007a). Using whole genome AFLP analysis, it was observed that the maximum recurrent genome content of only 86% was achieved in advance BC4DH generation which could not be improved in subsequent generations because of the "linkage drag" problem. Another significant observation of the study was the presence of a negative linkage between QTL alleles of low glucosinolate and seed yield (Ramchiary et al. 2007b; Bisht et al. 2009).

Transgenic approaches are faster, trustworthy and cost-effective alternative to modify the *Brassica* crops for low glucosinolate trait (Fig. 10.3) as the genetics of glucosinolate biosynthesis is much complex in *Brassica* crops. Silencing of candidate genes involved in glucosinolate biosynthetic pathway has been reported to reduce glucosinolate content in *B. napus* (Liu et al. 2011). The RNAi-mediated silencing of the *MAM* gene family in *B. napus* canola and rapeseed cultivars resulted in the reduction of total aliphatic glucosinolates and the total glucosinolate content. However, the *MAM* gene silencing in *B. napus* significantly induced the production of 2-propenyl glucosinolate. Targeting of transcription factors found to be more promising than targeting single genes as they control most of the genes involved in the pathway. *MYB28* has been identified as the major transcriptional regulator of aliphatic glucosinolate biosynthesis in *B. juncea* (Augustine et al. 2013a). Targeting the BjMYB28 through RNAi-based gene silencing resulted in the development of transgenic *B. juncea* lines having seed aliphatic glucosinolates as low as 11.26 μmol g^{-1} DW. The targeted silencing of *BjMYB28* homologs provided a significant reduction in the antinutritional aliphatic glucosinolates fractions, without altering the desirable non-aliphatic glucosinolate pool, both in leaves and seeds of transgenic plants. However, leaf glucosinolate was also found to be reduced in the transgenic lines (Augustine et al.

2013b). In a recent study, non-transgenic approach like TILLING was employed for the transport engineering of glucosinolates in oilseed *B. rapa* and *B. juncea* (Nour-Eldin et al. 2017). Since glucosinolates are synthesized in leaves and transported to seeds, this specifically reduces seed glucosinolate content, while maintaining a high level of foliar glucosinolates. The group mutated one of the seven and four of 12 GTR orthologs in *B. rapa* and *B. juncea*, respectively, and could achieve reduced glucosinolate levels in seeds by 60–70% in these two *Brassica* species.

10.6 Enriching the *Brassica* Crops with Glucoraphanin

10.6.1 Genetics of Glucoraphanin Accumulation

B. oleracea, including broccoli, Chinese kale, brussels sprouts and purple cauliflower contain high concentrations of glucoraphanin (Fahey et al. 1997; Liu et al. 2012). Even though rich in glucosinolates, there is only marginal or undetectable levels of glucoraphanin present in *B. juncea, B. napus* and in *B. rapa* which are among the widely cultivated *Brassica* crops around the globe (Kim et al. 2003; Padilla et al. 2007; Lou et al. 2008; Augustine and Bisht 2015). Though mainly used as an oilseed, young leaves of *B. juncea* and *B. napus* are consumed as vegetables in many parts of the world. Hence, it is essential to metabolically engineer these *Brassica* crops for high content of the nutritional giant glucoraphanin and low antinutritional glucosinolates.

Genetics of glucoraphanin accumulation in Brassicaceae has been investigated in detail (Giamoustaris and Mithen 1996; Kliebenstein et al. 2001a, b; Neal et al. 2010; Liu et al. 2014). Accumulation of glucoraphanin is genetically controlled by *GSL-AOP* locus which contains the *GSL-ALK* (*AOP2*) and *GSL-OHP* (*AOP3*) locus. Non-functional gene product of this locus is found associated with the accumulation of glucoraphanin. In *A. thaliana* ecotype Columbia, *AOP2* gene was shown to be marginally expressed which results in the accumulation of glucoraphanin (Neal et al. 2010). In broccoli, a non-functional *GSL-ALK* homolog has been identified which is associated with high-glucoraphanin accumulation. In *B. rapa,* three *AOP2* genes were identified which were found to be functional. Another recent study in polyploid *B. juncea* identified four functional homologs of *AOP2*. These findings clearly substantiate the reason for the absence of glucoraphanin in *B. rapa*, *B. juncea* and other related *Brassica* species (Li and Quiros 2003; Liu et al. 2014; Augustine and Bisht 2015). Both *AOP2* and *AOP3* encode a 2-oxoglutarate-dependent dioxygenase (2-ODD) enzyme. In arabidopsis, using enzyme assays of the heterologously expressed fusion protein, it has been suggested that the *AOP2* enzyme catalyzes the conversion of methylsulfinylalkyl glucosinolates to alkenyl glucosinolates and that the *AOP3* enzyme catalyzes the formation of hydroxyalkyl glucosinolates (Kliebenstein et al. 2001a, b). Further, it has been showed that the expression of functional *AOP2* correlates with the products of the *GSL-ALK* reaction and that expression of

AOP3 is completely associated with the products of the *GS-OHP* reaction. These observations conclude that the absence of either of these two enzymes can result in the accumulation of methylsulfinylalkyl glucosinolates. The study proved that *AOP2* and *AOP3* might have derived from the ancestral gene *AOP1* by gene duplication (Neal et al. 2010). The allelic variations of these genes play a key role in determining the type of glucosinolate accumulated in a particular *Brassica* species.

10.6.2 Development of Glucoraphanin Enriched Brassica

Enhancement of the beneficial glucoraphanin in *Brassica* crop through conventional breeding methods seems quite challenging, as the conventional breeding strategies are not simpler in polyploid crops like *B. napus* and *B. juncea* where gene multiplicity and redundancy result in complex genetic interactions. Faulkner et al. (1998) developed a hybrid between broccoli and *Brassica villosa* which accumulated significantly high concentrations of glucoraphanin in floral parts. In broccoli, high-glucoraphanin F1 hybrids were developed through genome introgression from the wild species, *Brassica villosa*. The high-glucoraphanin lines of Broccoli have been commercialized as Beneforte (Traka et al. 2013). Natural sources of mutations for *AOP2* loci are also limiting. Of late, there were no loss-of-function mutations in *AOP2* genes have been reported in *B. rapa* or in any other *Brassica* species except *B. oleracea.* Recently, Liu et al. (2017) identified natural non-functional mutations of two *BrAOP2* genes from "R-O-18" and then performed marker-assisted backcross breeding to substitute functional *BrAOP2* gene locus in "L58" with non-functional alleles to increase the beneficial glucosinolate (glucoraphanin) in *B. rapa.* Marker-assisted backcrossing was performed, and the screened backcross progenies with introgression of both non-functional *braop2.2* and *braop2.3* alleles showed a significant enhancement in the glucoraphanin content by 18 folds compared to the recurrent parent. Interestingly, introgression of a single non-functional allele, *braop2.2* or *braop2.3* did not alter the glucoraphanin content (Liu et al. 2017).

Knockdown of *GSL-ALK* gene has been shown to enhance glucoraphanin content in *B. napus* and Chinese kale (Liu et al. 2012; Qian et al. 2015). RNAi-based silencing of the *GSL-ALK* gene family in *B. napus* did not show significant alteration in the total glucosinolates content in the transgenic lines. However, the detrimental glucosinolate progoitrin was drastically reduced, and glucoraphanin was increased to a high concentration in the seeds of F1 progeny of a cross between *B. napus* ALK-RNAi line and a double haploid line of high glucosinolate containing rapeseed (Liu et al. 2012). However, the high-glucoraphanin trait in the F1 generation of *B. napus* showed a complex genetic control in advance segregating generations. Moreover, GNA was found decreased only when the gene silencing was very strong. In the antisense-mediated down regulation *of GSL-ALK* gene in Chinese kale, even though the glucoraphanin content was increased, a concomitant enhancement in gluconapin content was observed (Qian et al. 2015). In *B. juncea*, the constitutive silencing of *GSL-ALK* gene resulted in enhanced accumulation of glucoraphanin up

to 43.11 μmol g^{-1} DW in the seeds (Augustine and Bisht 2015). Along with the enhancement of glucoraphanin, a drastic decline in the concentrations of gluconapin and sinigrin as well as the total glucosinolate content was also observed. The level of glucoraphanin accumulated in the knockdown lines was found even higher than that of the glucoraphanin content reported in the florets of Benefort'e® broccoli. Other C_4 glucosinolate, glucoerucin which comes earlier in the pathway was found to be enhanced in the transgenic lines.

10.7 Conclusions and Future Directions

Development of low antinutritional glucosinolates and high-glucoraphanin traits are important breeding objectives in *Brassica* crops. Since the genetics of glucosinolate biosynthesis in model plant *A. thaliana* and few other *Brassica* crops are clearly documented, now it is time to translate this knowledge for *Brassica* improvement programs. Even though a few successful studies have been performed which could achieve high-glucoraphanin content in different *Brassica* crops through RNAi-mediated silencing or TILLING, the new generation of biotechnological tools like CRISPR/Cas9 seems to be promising in achieving these targets in lesser time with much more efficiency. This will also probably require less biosafety protocols and regulatory requirements.

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Chapter 11
Biotechnological Strategies for Development of Aflatoxin-Free Crops

Kalyani Prasad, Kiran Kumar Sharma and Pooja Bhatnagar-Mathur

Abstract Aflatoxins are secondary metabolites produced by the fungal genus *Aspergillus* (mainly *A. flavus* and *A. parasiticus*) that contaminate various agricultural commodities, but most prevalent in maize, groundnut, and cotton. Considered to be potent carcinogens and teratogens to humans and farm animals, aflatoxin contamination gets accentuated by hot and dry weather conditions, insect feeding and mechanical damage during and after harvest, and improper storage conditions. Growing global concerns about aflatoxin contamination have prompted search for effective control measures and specific regulations to limit exposure to these mycotoxins. Cultural practices include use of resistant varieties; control of insect pests, timely harvesting, proper drying, storage, sorting, and cleaning of harvested produce curtail aflatoxin contamination to some extent, and biological control strategies such as use of atoxigenic *A. flavus* strains have proven efficient in preventing infection by aflatoxin-producing strains. Genetic engineering for aflatoxin resistance through gene overexpression and recent development in area of transgenics through host-induced gene silencing of aflatoxin biosynthesis pathway genes have provided promising results in several crops such as cotton, corn, and groundnut. This book's chapter provides comprehensive overview on the various strategies and also updates the status of research to achieve aflatoxin resistance in crop plants. The role of various factors affecting aflatoxin contamination is also discussed that help to take appropriate measures for successful control of aflatoxin resistance. The availability of advanced molecular techniques, cutting edge tools and technologies provides greater potential to the development of markers and QTLs for aflatoxin resistance speeding up the development of durable aflatoxin-resistant varieties.

Keywords Aflatoxin · *Aspergillus* · Groundnut · Maize · Biotechnology

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11.1 Introduction

Aflatoxins (AFs) are hepatotoxic, cancer-causing secondary metabolites produced by three filamentous fungal species of *Aspergillus* including *A. parasiticus* Speare, *Aspergillus flavus* Link, and rarely by *A. nomius* Kurtzman (Ehrlich et al. 2003). Aflatoxin exposure in humans and livestock occurs through consumption of contaminated food and feed (Chulze 2010). These toxins suppress the immune system and retard growth in human and animals, particularly affecting the liver and digestive tract (Williams et al. 2004; Eaton and Groopman 2013). Based on the duration and amount of exposure, aflatoxins can lead to acute or chronic aflatoxicosis, while its prolonged exposure has been known to aggravate epidemics of AIDS, malaria, tuberculosis, and other diseases in many developing countries (Williams et al. 2005).

The aflatoxins are classified as AFB_1, AFB_2, AFG_1, and AFG_2 according to their absorption properties under ultraviolet light and molecular weight. While *A. parasiticus* can produce all the four toxins, *A. flavus* produces only AFB1 and AFB2. International Agency for Research on Cancer (IARC) categorized G- and B-type aflatoxins as Group 1 mutagens and AF-M1 as Group 2B (IARC 2015). According to a UN report, 25% of world food crops are contaminated by mycotoxins annually (Smith et al. 2016). Due to various risk factors, about five billion people from developing countries are frequently exposed to aflatoxin (Williams et al. 2004).

Aflatoxin contamination occurs more frequently in tropical and subtropical areas, usually in warm and dry weather conditions. *Aspergillus* infects crops not only at pre-harvest and post-harvest, but also during storage and transportation. Optimum growth conditions for *A. flavus* during post-harvest are between 25 and 30 °C and humidity levels of 0.99 aw, with the production of aflatoxin occurring optimally at 25 °C and 0.99 aw (Giorni et al. 2009). It affects broad range of agricultural commodities such as cereals (wheat, maize, rice, and barley), legumes (soybean, bean, and pulses), oilseeds (peanut, cottonseed, and coconut), spices (coriander, black pepper, chillies, turmeric, and ginger), nuts (pistachios, almonds, walnut) and dried fruits (figs, dates, dried apricot, and dried mulberries, etc.). Crops, particularly maize, peanut, cottonseed, pistachio, Brazil nuts, and coconut can be highly contaminated (Idris et al. 2010; Cornea et al. 2011); whereas, sorghum, oats, millet, wheat, barley, rice, soybean, cassava, beans, and pulses are occasionally contaminated. Other crops such as linseeds, melon seeds, cocoa beans are rarely contaminated (Bankole et al. 2010).

Despite the fact that aflatoxin contamination can cause significant losses to food in both developed and developing countries, the use of improved agricultural practices and strict legislative regulations imposed on food processing and marketing system have significantly reduced aflatoxin exposure in the developed countries. In contrast, developing countries either lack food safety regulations or regulatory compliance, besides the prevalence of conducive climatic and crop storage conditions for the *A. flavus* growth, thereby frequently contaminating the staple food crops (Hell et al. 2010).

Major causes of economic losses due to aflatoxin are yield, decreased crop value, animal and human health costs, export market, sampling and testing costs, costs to food processors, grocery markets and consumers (Wu 2004). Due to its serious health effects, aflatoxin was included in the Rapid Alert System for Food and Feed (RASFF) of the European Union in 2008. Aflatoxin enters the food chain through consumption of contaminated milk, eggs, meat, and their products (Bennett and Klich 2003).

Based on the significant correlation of AFB1 with liver cancer in parts of Africa, China, and South East Asia, aflatoxins are categorized as a Group I human carcinogen by the International Agency for Research on Cancer (Wogan 2000). Globally, it causes 40% of deaths and an estimated 5–30% of liver cancer, the most noteworthy occurrence being in Africa with 30% (http://www.xinhuanet.com). Besides causing health problems to humans, aflatoxin contamination of food crops leads to significant economic losses due to loss of crops and animals (Table 11.1).

Table 11.1 Economic losses due to aflatoxin

Crop	Economic losses (million US dollars)	Country	References
All crops	139	Africa–Senegal	http://www.xinhuanet.com
	38	Africa–Uganda	http://www.xinhuanet.com
	2	Africa–Gambia	http://www.xinhuanet.com
	750	Africa	Otsuki et al. (2001), Cardwell and Henry (2004)
	900	Philippines, Thailand, and Indonesia	Lubulwa and Davis (1994)
	85–100	USA	Yabe and Nakajima (2004)
Maize	52.1–1680	USA	Mitchell et al. (2016)
	225	USA and Canada	Vardon et al. (2003)
	15	USA-Texas	Robens et al. (2005)
	2	USA-Mississippi	Robens et al. (2005)
Peanut	26	USA and Canada	Coulibaly et al. (2008)
	120	USA	Wu (2004)
	75	Argentina	Wu (2004)
	40	Africa	Wu (2004)
	215	China	Wu (2004)

(continued)

Table 11.1 (continued)

Crop	Economic losses (million US dollars)	Country	References
	25	USA-Georgia	Robens et al. (2005)
Corn and peanut	47	World wide	Vardon et al. (2003)
Cotton	4	USA-Arizona	Robens et al. (2005)
	7	USA-Texas	Robens et al. (2005)
Walnut	38	USA-California	Robens et al. (2005)
Almonds	23	USA-California	
Ready-to-eat almonds, hazelnuts, and pistachios	9 Mio Euro	World wide	JECFA/WHO study (2006)
Feed crops	225	World wide	Vardon et al. (2003)

11.2 Aflatoxin and Food Safety Regulations

Several countries have imposed regulations to decrease dietary exposure to aflatoxin. Acute and chronic toxicity of aflatoxins necessitates enforcement of legislative limits on the presence of aflatoxin in about 100 countries, of which 61 have specific regulatory levels for total aflatoxins in foodstuffs, while 39 countries have regulations for aflatoxins in feedstuffs (Van Egmond et al. 2007). Aflatoxins are regulated in part per billion (ppb) ranges with the maximum permissible limit varying by country and proposed use of the commodity (Table 11.2).

Aflatoxins are relatively heat-stable and are not destroyed by normal food processing. Under favorable conditions, aflatoxin can be produced at temperature ranging from 11 to 40 °C, although 25–30 °C is the optimal range (Diener and Davis 1987). Despite several research and control measures, aflatoxin is as yet a noteworthy danger to agricultural crops leading to loss of millions of tons of crops annually. Several strategies used to control pre-harvest or post-harvest contamination with aflatoxin include good cultural practices, breeding for aflatoxin-resistant cultivars, biological control using atoxigenic fungal strains, harvesting crop at proper maturity, proper drying at harvest, and improved post-harvest storage methods.

This chapter highlights the progress on aflatoxin control approaches with a focus on three major crops maize, peanut, and cotton. Besides providing updates on the physical, chemical, and biological control strategies used for aflatoxin degradation in crops and during post-harvest, advanced molecular methods such as DNA microarray, qRT-PCR, and RNA-sequencing tools used to understand the nature of aflatoxin resistance have been addressed. In addition, we have reviewed the research activities on identifying more durable aflatoxin resistance genes, tagging of these genes or quantitative trait loci (QTLs) with molecular markers for marker-assisted selection (MAS) that are aimed to develop an integrated management program for aflatoxin resistance (Fig. 11.1).

Table 11.2 Regulatory limits for aflatoxins in different countries

Country	Commodity	Types of aflatoxins	Limit (ppb)
UK	Nuts, figs, and related products	B1	2–12
Europe	Groundnuts, nuts, dried fruit, and their products	Total, B1	4, 2
USA	In milk In food other than milk	M1 B1	0.5 20
Brazil	Groundnuts, corn, and other food products In milk	Total M1	20 0.5
Australia	All the food stuff except groundnut	B1	5
Germany	All food stuff	Total, B1	4, 2
India	Nuts, spices, cereals, and all other food products	Total, B1	30
China	Rice, sorghum, barley, nuts, etc.	B1	5–50
Indonesia	Corn feed	Total	15
	All foods	Total	35
	All foods	B1	20
	Peanut, corn, and their products	B1, Total	15, 20
	Dried milk and related products	M1	5
	Milk and milk products	M1	0.5
Japan	All food stuffs and rice	Total, B1	10
Kenya	All foodstuffs	Total, B1	10, 5
Malaysia	All foodstuffs	B1	35
Hong Kong	Peanut and peanut products Other foods	B1 B1	20 15
Vietnam	All foodstuffs	Total, M1	20, 0.5
Thailand	All foodstuffs	Total	20
Taiwan	Peanut, corn	Total	15
	Rice, sorghum, beans, wheat products, nuts, edible oils, and other foods	Total	10
	Infant foods	Total	0
	Milk powder	M1	5
	Fresh milk	M1	0.5
Srilanka	All Infant foods	Total M1	30 1
Singapore	All Infant foods	Total M1	5 0.5
Philippines	Human foods	Total	20
	Coconut, peanut products (export)	B1	20
	Milk	M1	0.5

(continued)

Table 11.2 (continued)

Country	Commodity	Types of aflatoxins	Limit (ppb)
South Korea	Grains, cereal products, dried fruits, meju, steamed rice, and baby foods	B1	10
	Baby foods	B1	0.1
	Grains, cereal products, dried fruits, meju, steamed rice, and baby foods	B2, G1, G2	15
	Raw milks and milk products prior to manufacturing processing	M1	0.5

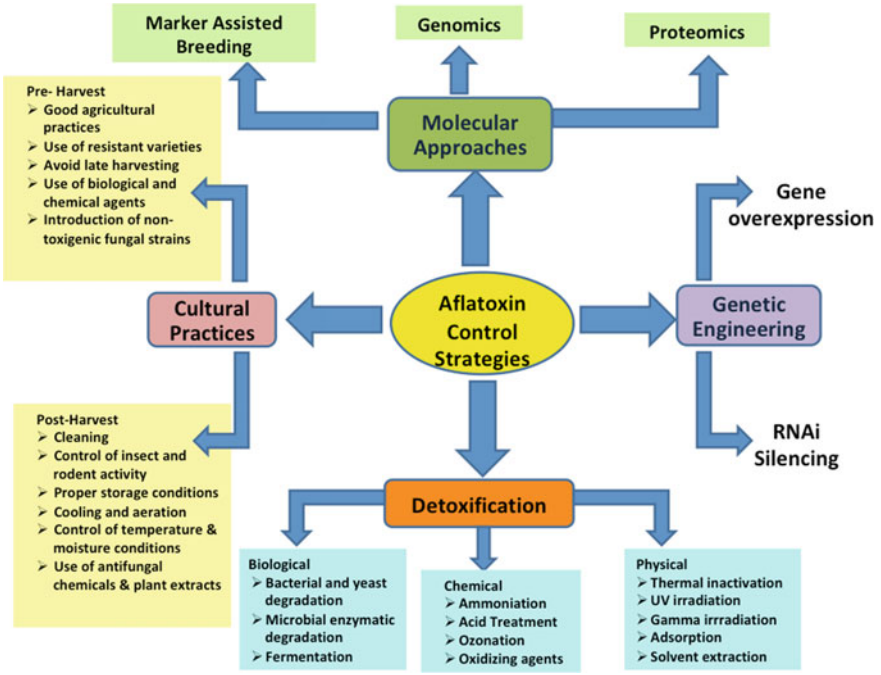

Fig. 11.1 Various approaches for aflatoxin resistance in crops

11.3 Genetics and Biochemistry of Aflatoxin Biosynthesis

Over thirteen species of the genus *Aspergillus* produce aflatoxins and their precursor sterigmatocystin (ST) (Varga et al. 2009). Structurally, aflatoxins (AFTs) have polycyclic structure derived from coumarin nucleus attached to bifuran group at one side, and on other side either a pentanone ring, or a six-membered lactone ring (Bennett and Klich 2003; Nakai et al. 2008). While B series aflatoxins have pentone ring, the G series have lactone ring. Aflatoxins have low molecular weight and are thermostable and highly carcinogenic in nature (Squire 1981; Sirot et al. 2013). Understanding

Table 11.3 Role of various cellular pathways involved or interfering with aflatoxin production

Gene	Function	References
VeA along with VelB and LaeA form the velvet protein complex	Coordinate primary and secondary metabolism	Bayram and Braus (2012)
mtfAI	Transcription factors	Ramamoorthy et al. (2013)
fcr3	Transcription factors	Shaaban et al. (2010)
AtfB, AP-1, and VeA	Regulate oxidative stress	Montibus et al. (2015)
rasA	Encode cellular signal mediators	Georgianna and Payne (2009)
G-protein receptors	Play role in signal transduction	Affeldt et al. (2014)
Oxylipins' biosynthetic genes	Stimulate aflatoxin production	Tsitsigiannis and Keller (2007)

aflatoxin biosynthesis is critical to find ways of eliminating or reducing its contamination in food grains. Significant progress has been made in identifying the role of several genes, enzymes, and regulatory processes involved in aflatoxin biosynthesis (Yu 2012).

The biosynthesis of AFB_1 involves a complex network with several interconnected metabolic pathways (Table 11.3) that play varied roles in fungal growth and aflatoxin production (Brodhagen and Keller 2006; Cary et al. 2012). This follows a series of oxidation–reduction reactions involving 25 enzymes, two regulatory proteins. and 15 intermediates (Yu et al. 2004). The genes are clustered within a 70-kb DNA region and transcriptional regulators such as *aflR* and *aflD* control the entire pathway (Yu et al. 2004). *AflR* and *AflS* are the regulatory genes where *AflR* encodes a sequence-specific DNA-binding binuclear zinc cluster (Zn(II)2Cys6) protein, where *AflS* is reported to be a potential co-activator of *AflR* (Meyers et al. 1998). Both these genes are required for transcriptional activation of aflatoxin structural genes (Payne et al. 1993). Besides *AflR* and *AflS*, the specific transcriptional regulators such as *Vea*, *Nsdc*, *LaeA,* or *Ap-1* positively regulates aflatoxin biosynthetic pathway (Chang et al. 2012; Reverberi et al. 2007).

Biosynthesis begins with the conversion of malonyl-CoA to a polyketide called noranthrone by the products of fatty acid synthase genes (*fas-1* and *fas-2*) controlled by *stcJ* and *stcK* and polyketide synthase gene *pksA* encoding *stcA* (Cary et al. 2000a, b, c). This intermediate is oxidized by the enzyme HypC to form the anthraquinone norsolorinic acid (NA), the first stable intermediate in aflatoxin biosynthesis (Ehrlich et al. 2010). NOR is converted to averantin (AVN) through the action of *nor-1*, *norA,* and *norB* genes, which is then converted to averufin (AVR) and later to versicolorin B (VERB) through the action of three genes including *cypX*, *moxY,* and *avfA* that catalyze individual steps. Two other genes; *ver1* and *verA* are required for the conversion of versicolorin A (VERA) to dimethyl-sterigmatocystin (DMST). The final step involves conversion of *O*-methylsterigmatocystin (OMST)

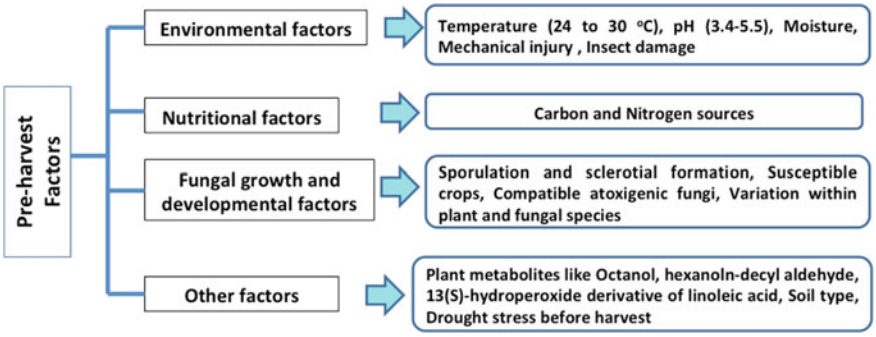

Fig. 11.2 Schematic representation of the aflatoxin biosynthetic pathway in *A. flavus*. The arrows indicate the pathway steps from previous precursor to the next intermediate toward the aflatoxins formation. The enzymes and the corresponding gene involved in each bioversion step are shown

or dihydro-*O*-methylsterigmatocystin (DHOMST) to aflatoxins in the presence of a NADPH-dependent cytochrome P-450 monooxygenase *ordA* (Yu et al. 2004). Sterigmatocystin is the penultimate precursor in the biosynthesis of aflatoxin (Fig. 11.2).

11.4 Factors that Affect Aflatoxin Biosynthesis

A number of environmental factors such as drought and temperature, nutritional factors such as carbon and nitrogen source, and developmental factors such as sporulation and sclerotia production influence *A. flavus* growth and subsequent aflatoxin contamination in crops (Fig. 11.3). Better understanding of all these factors aids in developing control strategies to reduce aflatoxin production in aflatoxigenic *A. flavus* species through regulation of conditions that favor fungal growth.

11.4.1 Nutritional Factors

Various nutritional factors such as carbon, nitrogen, amino acid, lipid, trace elements, and others affect aflatoxin production (Payne and Brown 1998; Cuero et al. 2003; Guo et al. 2005). Carbon and nitrogen source are the most prominent factors affecting aflatoxin biosynthesis (Adye and Mateles 1964; Bennett et al. 1979; Luchese and Harrigan 1993). *A. flavus* produces more aflatoxin on simple sugars such as glucose, sucrose, fructose, and maltose than on complex carbon sources such as starch, peptone, sorbose, or lactose (Buchanan and Lewis 1984; Payne and Brown 1998). Sugary kernels in corn showed more fungal growth and higher aflatoxin production than starchy kernels (Bressac et al. 1991). Location of sugar utilization cluster near

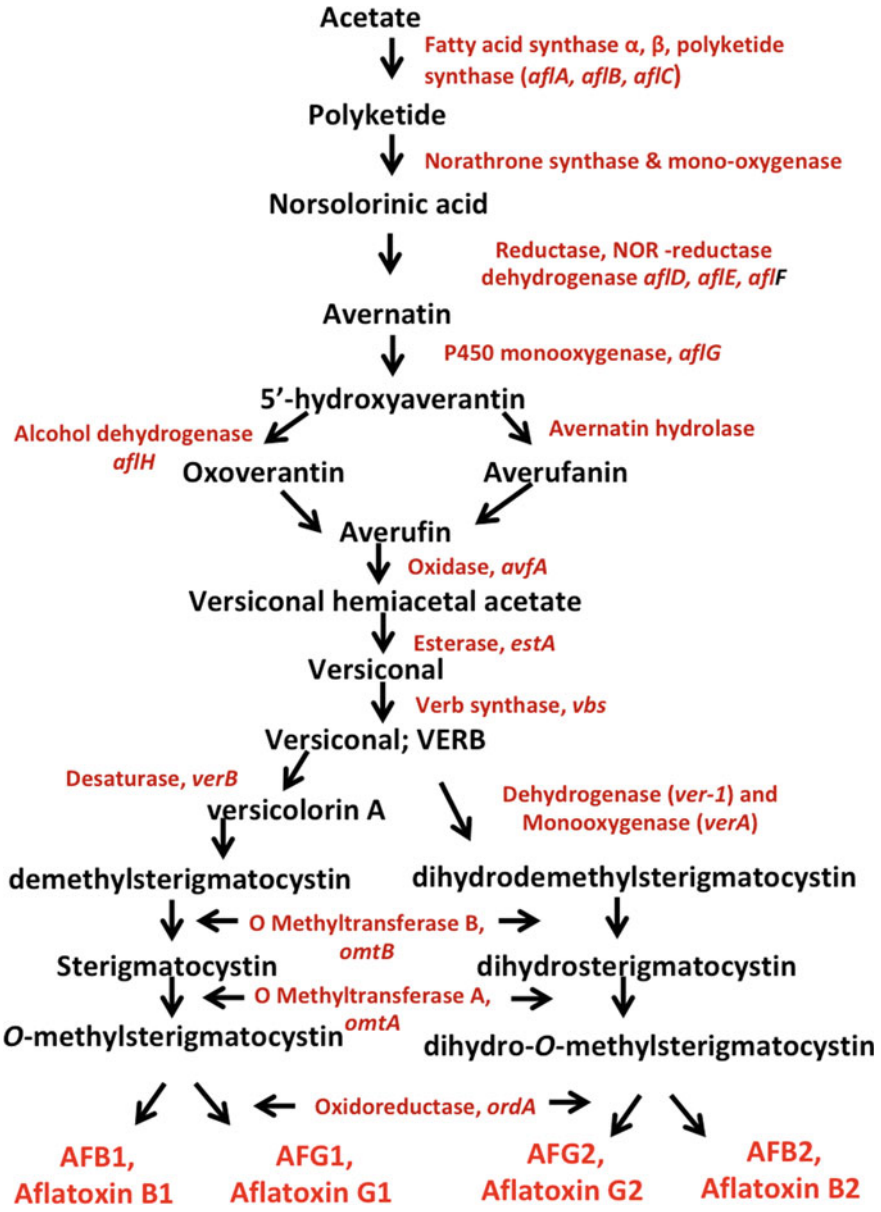

Fig. 11.3 Factors influencing aflatoxin production

to the aflatoxin gene cluster indicates a connection between the two gene clusters in carbohydrates processing that initiate aflatoxin production (Yu et al. 2000). Carbon sources such as glucose, maltotriose, maltose, and alpha-amylase activity induce aflatoxin production in *A. flavus* (Woloshuk et al. 1997). The nitrogen source can also affect the range of aflatoxin produced by *A. flavus* (Hsieh 1989). Complex organic nitrogen sources such as peptone, yeast extract, and casamino acids yielded higher level of aflatoxin compared to asparagine, aspartate, alanine, ammonium nitrate, ammonium nitrite, ammonium sulfate, glutamate, and glutamine, whereas sodium nitrate suppresses aflatoxin production (Davis et al. 1967; Reddy et al. 1979). Proline and asparagine produce higher aflatoxin than tryptophan or methionine (Payne and Hagler 1983). Several amino acids have varied effects on aflatoxin production; for example, tryptophan inhibits while tyrosine enhances aflatoxin production in *A. flavus* (Wilkinson et al. 2007).

Lipids in seeds provide a good carbon source to stimulate *A. flavus* growth and aflatoxin production. Seeds with polyunsaturated lipids produce more aflatoxin than seeds with monosaturated lipids (Fanelli et al. 1983, 1995; Fanelli and Fabbri 1989) because of the greater lipoperoxidation potential of polyunsaturated lipids. Trace elements such as zinc reportedly enhanced aflatoxin production in maize kernels, whereas manganese inhibited aflatoxin production (Failla et al. 1986; Hsieh 1989).

11.4.2 Environmental Factors

Temperature plays an important role in gene expression and aflatoxin production in *A. flavus* (Roy and Chourasia 1989; Obrian et al. 2007). While the optimum temperatures for the growth of *Aspergillus* spp. is 37 °C (Ogundero 1987), aflatoxin formation takes place at 30 °C (28–35 °C) and is totally inhibited when temperature increases to above 36 °C. Genomewide gene profiling through microarray, RT-PCR, and high-resolution studies using next-generation sequencing technologies revealed association of high temperature with a decrease in the expression of the aflatoxin pathway genes (Obrian et al. 2007; Yu et al. 2011; Gallo et al. 2016). Interaction of temperature with water activity (aw) affects the ratio of regulatory genes (*aflR*/*aflS*), which is directly proportional to the AFB1 production (Schmidt-Heydt et al. 2009, 2010). Regulatory gene a*flS* has been reported to be more affected by higher temperature than *aflR*. Alteration in the *aflS* to *aflR* ratio renders *aflR* non-functional for transcription activation.

Similarly, infield drought stress decreases water activity, thereby causing cracks in the peanut pod wall (Girdthai et al. 2010), increase silk cut in maize (Odvody et al. 1997), and develop hull cracking in pistachio (Doster and Michailides 1995; Hadavi 2005) leading to penetration of the *A. flavus* hyphae and increased aflatoxin contamination. Drought also curtails phytoalexin production that increases *A. flavus* infestation which may be due to depleted plant immunity (Wotton and Strange 1987; Dorner et al. 1989). Although, the available information makes it clear that heat and

drought stress increased crop susceptibility to *A. flavus* infestation, the process is still not clearly understood.

Studies on the effect of the pH of growth medium on aflatoxin production revealed that on ammonium-based acidic media, *A. flavus* produces more aflatoxin than in nitrate-based alkaline media (Cotty 1988). Transcriptional regulator *pacC* gene has shown to have a major role in pH homeostasis (Keller et al. 1997). In response to alkaline pH, *pacC* suppresses the transcription of acid-expressed gene *aflR*, thereby reducing aflatoxin formation (Espeso et al. 1993; Espeso and Arst 2000).

11.4.3 Fungal Growth and Development

Sporulation and sclerotia formation are genetically linked with secondary metabolism through a shared G-protein signaling pathway (Calvo et al. 2002). Secondary metabolite formation occurs after the fungus completes the initial growth phase and when spore formation starts (Trail et al. 1995; Hicks et al. 1997). Mutants lacking sporulation or some compounds that inhibit sporulation in *Aspergillus* also inhibit aflatoxin production (Reib 1982; Bennett and Papa 1988). During repeated sub-culturing, the aflatoxin-forming ability was gradually reduced accompanied with noticeable morphological changes such as increased vegetative mycelium, reduced vesicle number and diameter and disappearance of spherocytes (Torres et al. 1980).

11.4.4 Other Factors

Intracellular reactive oxygen species (ROS) in *A. flavus* produced by P450/monooxygenases and extracellular ROS in medium augmented with ROS production inducers like hydrogen peroxide and tert-butyl hydroperoxide trigger oxidative stress resulting in greater aflatoxin production. However, alleviation of oxidative stress using antioxidants such as hydrolysable tannins, ascorbic acid, and caffeic acid inhibits aflatoxin production (Jayashree et al. 2000; Bok and Keller 2004; Mahoney and Molyneux 2004; Kim et al. 2006). Heavier soils having higher water-holding capacity reduced the range of aflatoxin accumulation in peanut while light sandy soil was conducive for fungal growth (Torres et al. 2014). Several studies have also revealed that insect damage provides access point for fungal spores that accelerates the infection of *A. flavus* (Williams et al. 2002; Parsons and Munkvold 2010). Corn Bt-hybrids showed lower aflatoxin accumulation due to the resistance to borer (Williams et al. 2010).

11.5 Cultural Practices for Aflatoxin Management

Various control measures for aflatoxin contamination can target either pre- or post-harvest stages. Several cultural practices for the prevention of aflatoxins have been designed considering the various factors that affect fungal growth and aflatoxin production.

11.5.1 Pre-harvest

Pre-harvest measures helpful in yield enhancement can efficiently reduce the levels of aflatoxin. Several management options have shown potential toward reduction of aflatoxin contamination in field. These include use of resistant cultivars, plant spacing manipulation, altering sowing time, improving plant nutrition, reducing drought stress, crop rotation, tillage practices, and controlling other plant pathogens, weeds, and pests (Mixon et al. 1984; Rachaputi et al. 2002). Soil tests like soil pH and other parameters like mineral deficiencies that cause plant stress have been recommended. To avoid temperature effects and drought stress during seed development and maturation, crop planting at the optimal time is recommended. Soil amendments were found to be as effective in suppressing fungal infection and aflatoxin accumulation by 50–90% in peanut notably through the application of lime, crop residues, and compost, etc. (Waliyar and Adomou 2002; Waliyar 2006). Lime, a calcium source thickens the peanut cell wall and accelerates pod filling, whereas adding manure increases the growth of microbes that repress soil infections (Hell and Mutegi 2011). Soil types also affect the seed colonization levels with *A. flavus* and *A. parasitucus* (Graham 1982). Toxigenic fungi can rapidly proliferate in sandy soils and alfisols, particularly under dry conditions. Due to their high water-holding capacity, vertisols support lower aflatoxins contamination in peanut (Mehan et al. 1991). Although the application of gypsum to soil was shown to reduce aflatoxin contamination (Davidson et al. 1983), subsequent studies failed to substantiate this (Cole et al. 1985).

11.5.2 Harvest and Drying

The possibility of mycotoxin development and moisture content in storage is proportional (Lanyasunya et al. 2005). Moisture levels of 10–13% are considered safe for cereals, and it is recommended to dry the commodities as quickly as possible post-harvesting. In peanut, while mechanical or late harvesting increases the chances of aflatoxin contamination (Cole et al. 1995; Torres et al. 2014), over-mature or immature pods and humid harvesting conditions increased the chances of high levels of aflatoxin contamination (Kabak et al. 2006). Moreover, damage during digging and threshing peanut kernels makes them noticeably more susceptible to the fungal inva-

sion and subsequent aflatoxin contamination (Heathcote and Hibbert 1978). In maize too, severe grain loss occurs during storage because of extensive field drying (Borgemeister et al. 1998; Kaaya et al. 2006). Increase in aflatoxin by tenfold in 3-d period was noticed on storing harvested maize at high-moisture content (Hell et al. 2008). After harvesting, drying the grain to desirable moisture levels through sun drying or artificial drying reduces mycotoxin contamination. Several technologies employed to increase the grain drying efficacy and to lower the toxin contamination risk even under low-input conditions include drying platforms, drying outside the fields and on mats, etc. (Hell et al. 2008; Lutfy et al. 2008). However, because of the need for large capital investments, these dryers are not employed by African farmers. In Southeast Asia, these dryers have been used effectively to maintain rice quality and to reduce mycotoxin risk (Gummert et al. 2009).

11.5.3 Post-harvest Management

Post-harvest interventions like cleaning, sorting, rapid and proper drying, post-harvest pest control, and the use of natural or synthetic grain protectants reduce mycotoxins. Once crop gets infected under field conditions, the fungal growth continues with increasing vigor at post-harvest stage and also in storage. It is generally recommended to dry the crop immediately after harvest to safe moisture level of 10–13% for cereals such as corn and 7–8% for oilseeds such as peanut to prevent the fungal growth (Torres et al. 2014). Storage and feeding facilities should be decontaminated to prevent fungal growth on feed residues. Periodical evaluation of storage suitability is monitored with the help of CO_2 sensor.

Storage temperature plays a significant role in managing potential mycotoxin problems in the dried grain (Drying 1987). After drying, while the grains should be maintained at 1–4 °C for long-term storage as fungal metabolism is minimal at this temperature, for short-term storage grain temperature can be maintained between 10 and 15 °C during the summer. For maintaining the grain quality in storage, aeration is necessary to control temperature and to vaporize moisture in the bin. Regular inspection for pests, rodents, fungal growth temperature, moisture, off-odors, and warm spots should be observed timely. For example, weekly observations during the warm months and every 15 days during winter are needed. Since insect infestations in stored grains facilitate the growth of toxigenic fungi, controlling insects can help reduce the risk of molds and mycotoxins. An integrated approach is required to control the stored grain insects, which includes sanitation, good control of grain moisture and temperature, frequent monitoring, and fumigation (Holscher 2000).

In developing countries, the main cause of mycotoxin problems is insufficient storage facilities (Hell et al. 2000). After harvest, management of mycotoxins in high-moisture maize or silage rely on appropriate storage conditions like storing in anaerobic conditions by sealing. Toxigenic fungi do not proliferate and form mycotoxins under anaerobic conditions, even though the preformed mycotoxins will persist. Compared to dried grain, it is difficult to manage mycotoxin problem in

high-moisture maize or silage because the growth of toxigenic fungi is not visible until the symptoms are observed in livestock.

Traditional storage methods in Africa are categorized into temporary storage used for crop drying and permanent storage in the field or farm using containers made of plant materials. The stores are built in such a way so as to prevent insect, rodent infestation, and moisture from getting into the grains. Promoting the utilization of metal/cement bins poses challenges due to the associated costs. Many farmers store grains in polypropylene bags which are not airtight and facilitate fungal contamination and aflatoxin development (Udoh et al. 2000; Hell et al. 2000). Presently there are efforts to market improved hermetic storage bags in Africa based on triple bagging developed for cowpea (Murdock et al. 2012) which has been or is being tested for other commodities as well. Several studies have reported the application of Purdue Improved Crop Storage (PICS) bags to mitigate fungal growth and resulting aflatoxin contamination in maize and groundnut (Williams et al. 2014; Sudini et al. 2015).

11.6 Physical, Chemical and Biological Approaches for Tackling Aflatoxin Contamination

Despite several efforts, the pervasive nature of the *Aspergillus* in the field and during storage makes it very difficult to control fungal contamination. Hence, various physical, chemical, and biological methods (reviewed by Scherbakova et al. 2015) are essential to effectively detoxify aflatoxin contaminated food and feed.

Various physical strategies such as mechanical or electronic sorting, thermal inactivation, solvent extraction, bonding agents, ozonation, ultrasound treatment, inactivation through light or irradiation, and washing procedures can be used to decontaminate food or feed before ingestion. Nevertheless, some of these physical methods are expensive and may also remove or destroy important nutrients in commodities. In addition, since aflatoxins are relatively stable at high temperatures (80–121 °C), they cannot be removed by usual cooking methods such as frying, boiling, or pasteurization (Samarajeewa et al. 1990; Murphy et al. 2006). In addition, most of the physical methods are time-consuming and result in only partial removal of aflatoxin (Wang et al. 2011).

Various chemical agents such as acids (hydrochloric acid), bases (ammonia, hydrated oxide), oxidizing reagents (hydrogen peroxide, ozone), reducing agents, and chlorinating agents can be applied by fumigation, immersion, mixing, and packing to degrade mycotoxins. Acid treatment with hydrochloric acid destroys AFB1 levels by 19.3% within 24 h following the treatment (Doyle et al. 1982). Ammoniation utilizing gaseous ammonia or ammonium chloride reduced aflatoxin concentrations by more than 75% (Burgos-Hernández et al. 2002). However, both ammonia and hydrochloric acid reduce the nutritional value of the treated commodities, thereby limiting their use. Only limited chemical agents were effective at the laboratory level

and need to be validated before commercialization. Prevention of aflatoxin contamination in stored grains using chemical strategies has recently been reviewed (Nesci et al. 2016; Alberts et al. 2017).

Biological agents control aflatoxins through microbial degradation of the toxic metabolites or by antifungal activity. Microbial degradation targets furofuran and lactone rings of aflatoxin are product-specific, have mild reactions conditions, and are feasible compared to physical and chemical methods when applied in food and feed industries (Kolosova and Stroka 2011). However, microbial degradation of aflatoxins also causes undesirable reductions in quality of the commodity besides requiring regulatory approvals. Microbes directed at preventing infection that are environmentally acceptable are potentially more useful since these have a longer period of efficacy and are more readily distributed than the agrochemicals. Various studies reported the use of microbes such as bacteria, yeasts, and non-toxigenic (atoxigenic) strains of the causal organisms for biological control of aflatoxin contamination (reviewed by Verheecke et al. 2016). However, only the use of non-toxigenic strains has reached the commercial stage.

11.6.1 Competitive Fungal Control Strategy

Among various biocontrol agents, the use of competitive non-toxigenic strains of *A. flavus* and/or *A. parasiticus* for biological control of pre-harvest aflatoxin contamination in crops has been largely effective and been adopted. Competition between toxigenic and non-toxigenic strains of *A. flavus* for the same ecological niche provides this basis for biological control. The effectiveness of atoxigenic *A. flavus* strains depends on their predominant asexual nature, genetic stability and aggressive as competitors together with their inability to recombine with native toxigenic strains (Abbas et al. 2011; Ehrlich and Cotty 2004). Approaches and techniques such as tandomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellite markers have been used to differentiate *A. flavus* strains and to assess the competitive ability of atoxigenic strains (Tran-Dinh et al. 2018).

Various factors such as site of application, formulation, inoculum rate, and time of application affect the ability of non-aflatoxigenic fungi in reducing aflatoxin contamination. To competitively exclude toxigenic strains, atoxigenic strains must be applied to the same niche as *Aspergilli* toxigenic strains, mostly to soil of susceptible crops such as peanuts, cotton, and corn. Although, soil application of non-toxigenic strains reduces the dust-borne wild-type aflatoxigenic *A. flavus* spores from reaching the silks in maize through competitive exclusion in soil. However, for effective biocontrol, inoculum of non-toxigenic strain should be applied closer to infection site such as silks and leaves in corn, and foliar plant parts in treenuts such as pistachio, almonds, and walnuts (Dorner 2009). Among the various formulations, use of organic grain matrix prepared by sterilizing grain (sorghum, wheat, rice) or by coating the surface of the grain with conidia of the strain is the most effective method (Dorner 2004). Further, bioplastic-based granular formulation of non-aflatoxigenic *A. flavus*

was developed and successfully tested under field conditions as soil and foliar applications (Accinelli et al. 2009, 2016). In addition, continued application of biocontrol agent seasonally is required to maintain high population of atoxigenic strains compared to toxigenic strains for long-term control of aflatoxin contamination (Dorner et al. 1998; Pitt and Hocking 2006). Depending on environmental variations such as soil temperature, *Aspergillus* spp. population in soil, and the crop cultivars, the suitable time for application of non-toxigenic strains has to be optimized in each region (Dorner et al. 1992, 1998; Dorner 2004). Although studies have reported that use of pre-emergent herbicide did not affect the growth of the non-toxigenic *Aspergillus* strains (Pitt and Hocking 2006), direct exposure to herbicides has reduced spore production of AF36 (Garber and Cotty 2006), thereby suggesting that non-toxigenic strains should be applied after all herbicide applications have completed. The efficacy of atoxigenic *A. flavus* as biocontrol agent has been reported in peanut, corn, and cotton in several countries (Table 11.4). A total of two non-toxigenic fungi have been registered with EPA and one under registration at African biopesticide regulatory agencies for control of toxigenic *A. flavus* (Table 11.5). Most of these are currently

Table 11.4 Efficacy of non-toxigenic *Aspergillus* strains as biocontrol agent

Crop	Strain	Aflatoxin reduction (%)		Country	References
		In vitro	Field		
Maize	A2085 AF-X1™			Italy	Mauro et al. (2018)
	A2066, A2085, A2090, A2103, A2321	61–90		Italy	Mauro et al. (2015)
	La3303, La3304, La3279, Kal6127		67–95	Nigeria	Atehnkeng et al. (2014)
	AF36, K49, NRRL 21882, La3279, F3W4 (NRRL 30796) and K54 (NRRL 58987)		99.3		Jane et al. (2012)
	K49 (NRRL 30797), NRRL 21882 (Afla-Guard)		83–98	USA	Abbas et al. (2012)
	La3328, La3279		70.1–99.9	Nigeria	Atehnkeng et al. (2008)
	BS07, MN1, TOϕ, 696A, MAM13	78.2–80.2		Italy	Degola et al. (2011)
	NRRL 21882 *A. flavus* aggressive strain		66–87	USA	Dorner et al. (1999)
	More than 12 native *A. flavus* strains	>80		Kenya	Probst et al. (2011)

(continued)

Table 11.4 (continued)

Crop	Strain	Aflatoxin reduction (%)		Country	References
		In vitro	Field		
	K49 and CT3		37–94	USA	Abbas et al. (2006)
	CT3 and K49 (NRRL 30797)			USA	Abbas et al. (2006)
	BN30			Africa	Cardwell and Henry (2004)
Peanut	AFGS5 or AFGS12				Mallikarjunaiah et al. (2017)
	AFGS5 and AFGS12		0–2 ppb		Navya et al. (2016)
	GD-15	33.5–99.6		China	Zhou et al. (2015)
	AR27, AR100G, AFCHG2		78.36–89.55	Argentina	Zanon et al. (2016)
	AFCHG2			Argentina	Zanon et al. (2013)
	NRRL 21882 Afla-guard®		75	USA	Dorner (2009)
	AF051		99	China	Yin et al. (2008)
	NRRL 21882		91.6	USA	Dorner and Horn (2007)
	>2 strains		43–98	Australia	Pitt and Hocking (2006)
	NRRL 21368		89–95	USA	Dorner et al. (2003)
	Color mutant, NRRL 21368	78.2–99.9		USA	Dorner et al. (1998)
Cotton	AF 36 (NRRL 18543)		20–88	USA	Cotty and Bhatnagar (1994)

sold commercially as one or more products.

The application of biocontrol products with atoxigenic *A. flavus* active ingredient is one of the most promising and cost-effective method to reduce aflatoxin content in several crops. However, several potential challenges need to be addressed before commercialization. Various factors such as sexual recombination (Razzaghi-Abyaneh et al. 2014) and reassortment of genes (Olarte et al. 2012) within the aflatoxin gene clusters of *A. parasiticus* and *A. flavus* populations may reduce the effectiveness of atoxigenic *A. flavus* as biocontrol agent. Further, there may be a risk of production of novel *A. flavus* phenotypes, resulting in greater diversity in the field (Fisher and Henk 2012). Hence, unraveling the genetic variations among *A. flavus* strains is essential to design efficient biocontrol strategy (Ehrlich 2014).

Table 11.5 List of commercialized non-toxigenic *Aspergillus* strains as biocontrol agent

Product	Crop	Developed by	Registered for commercial use in countries	Manufacturer or distributor	References
AF36 contain single atoxigenic VCG NRRL 18543	Cotton seed, maize, and pistachio	USDA-ARS	Arizona, Texas, and South California	Arizona Cotton Research and Protection Council, USA	Cotty (2006), Cotty and Jaime-Garcia (2007), Doster et al. (2014)
Afla-Guard contain single atoxigenic VCG NRRL 21882	Maize and peanut	USDA-ARS	USA	Circle One Global, USA Syngenta	Dorner (2004), Dorner and Lamb (2006)
Afla-safe constituted with four unique atoxigenic VCGs	Maize and peanut	International Institute of Tropical Agriculture (IITA) and the USDA-ARS	Africa (Kenya, Nigeria, Senegal, and Gambia)	SODEFITEX (www.sodefitex.sn), a private company based in Senegal	Bandyopadhyay and Cotty (2013), Grace et al. 2015

11.7 Host Resistance

Comprehensive understanding of host plant resistance would be an efficacious approach to speed up the genetic improvement of aflatoxin-resistant cultivars (McMillian et al. 1993; Cole et al. 1995; Williams and Windham 2001; Naidoo et al. 2002; Guo et al. 2006, 2007; Holbrook et al. 2008). Despite numerous efforts, limited progress in aflatoxin resistance breeding is seen due to lack of resistance levels for pre-harvest seed infection, in vitro seed colonization (IVSC), and aflatoxin formation by *A. flavus*. Major hurdles are performance variation due to high genotype × environment interaction, dearth of reliable screening techniques, and limitations in understanding the genetics of resistance. Breeding efforts to attain high level of combined resistance to seed infection, IVSC, and aflatoxin production do not produce intended results. Due to lack of accessibility of biomarkers, transfer of polygenic aflatoxin resistance from inbred lines to commercial elite varieties with desirable agronomic traits has been a very slow process (Menkir et al. 2008).

11.7.1 Breeding for Resistance

11.7.1.1 Peanut

Development of peanut cultivars with aflatoxin resistance requires knowledge about genetic variation responsible for resistance in the germ pool and reliable screening techniques. Resistance to aflatoxin-producing fungi in peanut has been divided into three types including resistance to (a) infection to pod wall, (b) seed coat infection and colonization, and (c) aflatoxin formation by cotyledons. Pod wall serves as the first physical barrier to the fungal entry, and resistance is imputed to structure of pod shell (Zambettakis 1975). The second barrier is seed coat for which resistance depends on the density and thickness of palisade layers, wax layers, and lack of cavities and fissures (Zambettakis 1975; Pettit et al. 1989; Liang et al. 2006). There are conflicting reports concerning the effect of fungistatic phenolic compounds in providing resistance at the seed coat level (Upadhyaya et al. 2002). Microscopic fissures in seed coat can be formed at the pod development stage because of stresses caused by heat and water activity (Dickens 1977; Glueck et al. 1977). Few of the methanol-extracted and water soluble tannins from peanut testa and cotyledons notably inhibit *A. parasiticus* and reduce aflatoxin (Azaizeh et al. 1990). Insects and nematode damage to the developing pods in soil provide access points to the fungal pathogens. In case the seed testa is damaged, seed coat-related resistance will have limited value. Since cotyledons are the ultimate feeding site for fungi where aflatoxins are produced, aflatoxin production will reduce when cotyledons do not support the pathogen growth. Seeds of resistant cultivars having high total phenol and protein content showed higher resistance to *A. flavus* compared to susceptible cultivars having greater amount of total

sugars (Premlata et al. 1990) indicating that the phenolic compounds and proteins play an important role in peanut aflatoxin resistance.

Screening peanut genotypes for resistance to in vitro seed colonization (IVSC) is constrained by a number of factors such as significance of genotype to environment interactions (Mixon 1986; Mehan et al. 1983), poor correlation of in vitro seed colonization with field colonization, and aflatoxin accumulation in the field (Will et al. 1994, Blankenship et al. 1985; Kisyombe et al. 1985; Anderson et al. 1995). Although several workers have reported significant correlation between IVSC and field tolerance, the relationship is inconsistent (Mehan 1989). Under extended drought conditions, peanut genotypes maintained with high-moisture content in kernel showed enhanced resistance and produced low aflatoxin (Cole et al. 1993). However, no consistent relationship was obtained between IVSC and aflatoxin accumulation in peanut, and aflatoxin accumulation was inversely proportional with relative water content (RWC), pod wall integrity and pod wall moisture content during harvest (Sudhakar et al. 2007). Zambettakis et al. (1981) attributed pod infection to variation in shell structure. Priyadarshini and Tulpule (1978) recorded no correlation between fungal colonization and aflatoxin formation and concluded that aflatoxin production depends on many other environmental factors apart from *A. flavus* infection. Therefore, the range of *A. flavus* seed infection cannot be absolutely correlated with the aflatoxin formation (Davidson et al. 1982).

A screening technique which provides environmental control and results in low coefficient of variation in data was developed to identify genotypes with resistance to aflatoxin accumulation when exposed to post-harvest conditions favorable to fungal growth and aflatoxin synthesis (Xue et al. 2004a, b). This study showed that different strains of *Aspergillus* produce varying ranges of aflatoxin in peanut genotypes. Hence, the use of mixture of several aflatoxigenic strains of *A. flavus* and *A. parasiticus* was suggested to identify genotypes with stable and low aflatoxin contamination. More recently, in vitro screening methods have been developed to screen transgenic events that normally produce few seeds (Arias et al. 2015; Sharma et al. 2017). To limit the source of variation in analysis, both the testa and embryo were removed and half cotyledon used for *A. flavus* inoculation, and subsequently aflatoxin content measured at 72 h post-inoculation by ELISA and HPLC. This method allowed the proliferating fungi to reach the cotyledons and ultimately result in aflatoxin formation in the seed. Hence, it is essential to study resistance level offered by cotyledons to prevent aflatoxin buildup. This study provides a robust approach to screen transgenic decoated seeds in a non-destructive manner using minimal tissues. This method could also be used in breeding programmes to screen peanut germplasm for aflatoxin resistance.

Due to poor performance of in vitro selected genotypes under field conditions, the research focus has been moved to identify resistant sources to pre-harvest infection and aflatoxin contamination. Holbrook et al. (1994) established a large-scale field screening technique to evaluate field resistance to pre-harvest aflatoxin contamination, utilizing sub-surface irrigation in the desert to allow exposure to late season drought stress in pod zone while maintaining the plant alive. Initial field tests in desert environment had reported the death of peanut plants and dehydration of seeds in soil before contamination could start. By using sub-surface irrigation, mean

aflatoxin contamination increased by over 100% where the coefficient of variation (CV) reduced by over 50%, besides reducing the percentage of escapes by over 90%. Anderson et al. (1996) later developed a screening technique that could be used in standard greenhouse facilities. Various experiments have been conducted to optimize the adequate drought stress and level of fungal infections required for aflatoxin screening. Maintaining the pod zone dry and limiting the moisture to the root zone increase the amount of pre-harvest aflatoxin contamination.

The normal method for inoculating peanut using water as a carrier (conidial water suspension of *A. flavus*) at mid-bloom provides higher inoculum load, although the *Aspergillus* population in soil reduced shortly after inoculation. However, in subsequent studies, using sterilized corn seed inoculated with fungus maintained stable *Aspergillus* populations in soil at harvest compared to the former (Will et al. 1994). Holbrook et al. (1993) preliminary screened all the germplasm accessions in core collection first in single environment, and the lines that showed lower contamination levels were subsequently screened in two environments. Following this method, 19 US peanut core accessions with reduced levels of aflatoxin contamination were identified in different environments (Holbrook et al. 2009). These identified resistant sources were used in a hybridization program for introgression of resistance gene into agronomically acceptable cultivars.

To evaluate pre-harvest field infection and aflatoxin content in peanut germplasm, microsick plots infested with *A. flavus* were designed at ICRISAT (Fig. 11.4). This screening technique prevents escapes, reduces coefficient of variation, and ensures higher and more consistent contamination (Bhatnagar-Mathur et al. unpublished). Using these concrete structures, late season drought was imposed in the pod zone by retaining high soil temperature of 28–30 °C conducive for fungal growth and contamination, while maintaining the plant alive through watering only in root zones This screening technique could be useful to find aflatoxin resistance sources in peanut germplasm and transgenics through rigorous evaluation under simulated field conditions (Bhatnagar-Mathur et al. unpublished results).

A complete list of peanut genotypes reported from different countries to have aflatoxin resistance is given in Table 11.6. Although, a few reports are available on mini core collections for *A. flavus*/aflatoxin resistance screening (Yugandhar 2005; Kusuma et al. 2007; ICRISAT 2009), details on the resistant sources in the ICRISAT mini core collection are not available. Waliyar et al. (2016) reported seven best accessions that over a six years period (2008–2013) consistently accumulated low levels of aflatoxin (<4 μg/kg), ICGs 14630, 13603, 3584, 1415, 5195, 6888, and 6703. Although several sources of aflatoxin resistance in peanut have been reported, none of these showed immunity toward infection by *Aspergillus* spp. and/or aflatoxin accumulation. However, some of the identified potential sources could be used to understand the resistant mechanisms for developing resistant varieties or introgressing resistance in popular released varieties with the goal to develop high-yielding varieties adapted to different agro-ecosystems with enhanced resistance to aflatoxin.

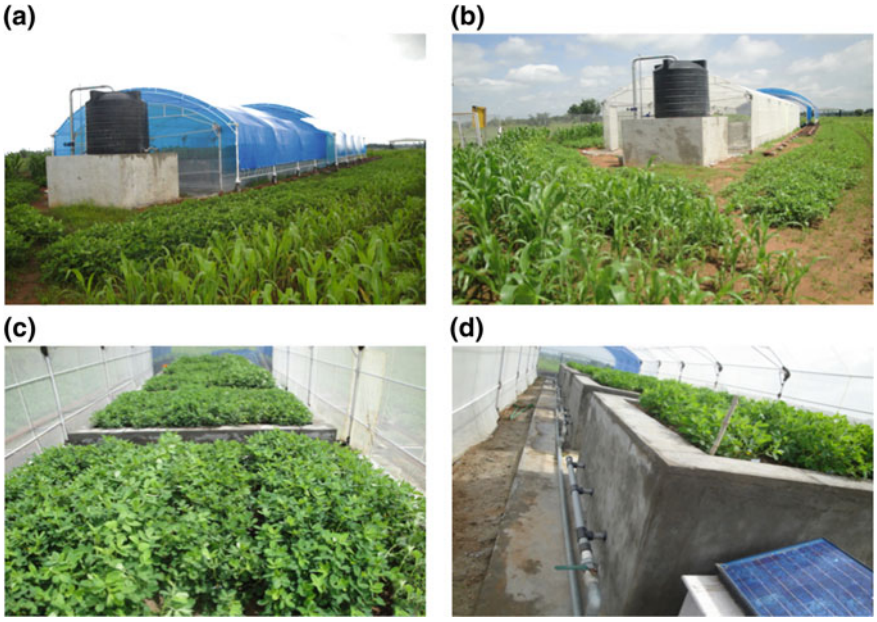

Fig. 11.4 Microsick plot design at ICRISAT (**a**). Overlapping rain out shelter (**b**). Rain out shelter with biosafety net (**c**). Sixty days old peanut crop in microsick plots (**d**). Water for root zone only from 60 days after sowing

11.7.1.2 Maize

Effective screening techniques to identify aflatoxin resistance would be valuable for maize breeding programs. Various procedure for inoculating maize ears with spore suspension under field conditions and techniques for evaluating resistance to fungal infection and aflatoxin content have been periodically updated over the years (Scott and Zummo 1988, 1990a, b, 1994; Zummo and Scott 1989; Windham et al. 2003; Williams et al. 2003). In maize, three factors that contribute to resistance to *A. flavus* and aflatoxin accumulation include pericarp structure, thickness and surface wax, and sub-pericarp components like preformed or induced proteins inhibiting fungal growth or aflatoxin production. Although few sources of maize kernel pericarp resistance (Guo et al. 1995; Russin et al. 1997) and sub-pericarp resistance (Brown et al. 1993, 1995) have been identified, still it is difficult to attain high levels of genetic resistance to aflatoxin in maize. Major limitations are inconsistent results due to high GxE interaction, lack of reliable and rapid inoculation techniques, polygenic resistance, low heritability, lack of appropriate resistant control, and the expensive detection techniques, especially for aflatoxin levels. Development of laboratory kernel screening assay (KSA) and use of β-glucuronidase (GUS) or green fluorescent protein (GFP) expressing *A. flavus* strain to monitor the degree of fungal infection in kernels should be very useful for rapid identification of resistant corn genotypes.

Table 11.6 List of aflatoxin-resistant peanut genotypes

Resistant Genotypes	Screening technique	Aflatoxin levels	Country of origin	References
US 26 (PI 246388)	IVSCAF	Reduced aflatoxin production	USA	Rao and Tulpule (1967)
Asiriya Mwitunde	Field trials	Reduced aflatoxin production	ICRISAT, India	Kulkarni et al. (1967)
PI 337409 and PI 337394F	IVSCAF	5 and 9% seed infection respectively	Argentina	Mixon and Rogers (1973)
Darou IV and Shulamit	*A. flavus* infection in pod	Reduced level of pod infection	Senegal	Zambettakis (1975)
PI 337409, PI 337394F, Ah 7223, UF 71513, J 11, U 4-47-7, Monir 240-30 Var, 27, and Faizpur	IVSCAF	Up to 15% seed colonization	USA	Mehan and McDonald (1980)
J11, PI 337409, and PI 337394F	IVSCAF	8–15% seed colonization	ICRISAT, Senegal and India	Zambettakis et al. (1981) and Kisyombe et al. (1985)
UF 7 1513, Ah 7223, J11, PI 337409, and PI 337394F	IVSCAF and field trials	15% seed infection and aflatoxin content up to 100 µg/kg seed	ICRISAT, India	Mehan and McDonald (1984)
VRR 245 and U 4-7-5 and	Field infection of seed by *A. flavus*, and for aflatoxin contamination	aflatoxin Bl 7–10 µg/kg seed	ICRISAT	Mehan et al. (1986a)
J11, PI 337 409, and PI 337394F	Natural infection by *A. flavus*	Lower aflatoxin production	ICRISAT	Mehan et al. (1986b)
AR 1, AR 2, AR 3, and AR 4, GFA 1, and GFA 2	IVSCAF	8–13% seed colonization	Argentina	Mixon (1986)
GFA 1, GFA 2, PI 337394F and PI 337409	Natural infection by *A. flavus*	Lower aflatoxin production	Argentina	Mixon (1980, 1986)

(continued)

Table 11.6 (continued)

Resistant Genotypes	Screening technique	Aflatoxin levels	Country of origin	References
PI 337394F, J11, UF 71 513, U 4-47-7 and Ah 7223	Field infection of seed by *A. flavus*, and for aflatoxin contamination	0.8–2% fungal infection and lower aflatoxin Bl content of 9–23 μg/kg seed	Four drought-prone sites in Andhra Pradesh, India	Mehan et al. (1987)
J 11	Field trials	Lower	North Carolina, USA, and India	Kisyombe et al. (1985), Mehan et al. (1987)
GFA 1, GFA 2, PI 337394 F, PI 337409, UF 71513, Ah 7223, J 11, Var 27, U 4-47-7, Monir 240-30 and Faizpur	IVSCAF	0.8–1.5%	ICRISAT, India	Mehan (1989)
CVS 55-437, 73-33 and 73-30	IVSCAF	Moderate to high levels of fungal infection	ICRISAT	Waliyar and Bockelee-Morvan (1989)
Tifton 8 and Southern Runner	Field trials	Lower aflatoxin production	National Peanut Research Laboratory, USA	Wilson et al. (1990), Cole et al. (1993)
ICG 3336, ICG 3700, ICG 1326, ICG 3263, ICG 7633 and ICG 4749	Field screening in sick plot under imposed drought conditions	Lower aflatoxin production	ICRISAT, Senegal and India	Mehan et al. (1991)
ICG 4749, ICG 1326, ICG 3263, ICG 3700, ICG 4888, ICG 9407 and ICG 7633	IVSCAF	Lower aflatoxin production	ICRISAT, India and Senegal	Mehan et al. (1991)
55-437, J 11, PI1337394 F, ICGV 87084, ICGV 87110, and ICGV 87094	Field trials	5–37% fungal infection and aflatoxin content ranging from 1 to 450 ppb	Niger, Senegal, and Burkina Faso in West Africa	Waliyar et al. (1994)

(continued)

Table 11.6 (continued)

Resistant Genotypes	Screening technique	Aflatoxin levels	Country of origin	References
ICGV 89104, ICGV 88145, ICGV 91278, ICGV 91284, and ICGV 91283	Field trials	Lower aflatoxin production	India	Rao et al. (1995), Upadhyaya et al. (2001)
ICG 1323, ICG 1326, ICG 1122, ICG 1173, ICG 3263, ICG 3267, ICG 1859, ICG 1994, ICG 3336, ICG 3700, ICG 4589, ICG 4749, ICG 9610, ICG 10020, ICG 10094, ICG 10933 ICG 4888, ICG 7412, ICG 7633, ICG 8666, ICG 9407	Field screening in sick plot under drought stress	<2% seed infection	ICRISAT Center, India	Singh et al. (1997)
A.pusilla [ICG 13212 (PI 497572, VSW 6773)], A. chiquitana Krapov., W.C. Gregory and C. E.Simpson [ICG 11560 (PI 476004, KSSC 36025)], *A. triseminata.* Krapov., ICG 14875 (VfaPzSv 130800)] and W.C Gregory [ICG8131 (PI 338449, GK 12922)	IVSCAF and aflatoxin production	0–100 μg/kg seed	ICRISAT, India	Thakur et al. (2000)

(continued)

Table 11.6 (continued)

Resistant Genotypes	Screening technique	Aflatoxin levels	Country of origin	References
ICGV 95440, ICGV 95422, UF 71315, ICGV 94435, ICGV 94434, ICGV 94433, and ICGV 95435	Field trials	<10 μg/kg seed	India	Zhou et al. (2002)
PI 590325, PI 590299, PI 290626, and PI 337409	Modified IVSCAF with seed testa removed and aflatoxin production quantified	Showed consistently lower aflatoxin production	USA	Xue et al. (2004a)
A. duranensis Krapov, PI 475997 *A.cardenasii* Krapov. and W. C. Gregory accessions PI 468200, PI 468319, and PI 262141 and PI 262133	Modified IVSCAF with seed testa removed and aflatoxin production quantified	Showed consistently lower aflatoxin production	USA	Xue et al. (2004b)
Yueyou 9	IVSCAF	Less than 19% seed infection	China	Li (2006)
G845 and G8	IVSCAF	Lower aflatoxin production	China	Jiang et al. (2006)
Taishan Zhengzhu and Zhonghua 6	IVSCAF and field trials	Lower aflatoxin production	China	Lei et al. (2004) and Liao et al. (2003)
N1211 and N1322	Field trial	Lower aflatoxin production	China	Xiao et al. (1999)
Zhonghua 6	Field trial	Lower aflatoxin production	China	Liao et al. (2009)
ICG 12625 and ICG 2381, ICG 12697	Field trials		ICRISAT, India	Upadhyaya et al. (2013)
ICGs 1415, 13,603, 14,630, 3584, 5195, 6888 and 6703	Field trials	<4 μg/kg seed	ICRISAT, India	Waliyar et al. (2016)

KSA can be used for preliminary evaluation of germplasm to identify pericarp and sub-pericarp level of resistance (Brown et al. 1993, 1995). However, field trials are indispensable for the final substantiation of resistance.

Several reports show that low fungal growth correlates with lower aflatoxin production in maize (Brown et al. 2001; Magbanua et al. 2013; Fountain et al. 2015; Wang et al. 2016a, b; Garrido-Bazan et al. 2018). This restricted *A. flavus* growth in resistant cultivar is attributed to preform host barriers such as pericarp wax, cutin, and polyphenolic compounds impeding the fungus from passing through the pericarp to colonize the seed (pericarp resistance) or presence of highly cross-linked lignin. Wang et al. (2016a, b) showed that genes linked with growth of mycelium, conidiation, and aflatoxin biosynthesis were upregulated in aflatoxin susceptible peanut lines compared with resistant lines and showed that aflatoxin production was correlated with fungal establishment and proliferation. However, other reports have shown that resistance to *A. flavus* and aflatoxin accumulation is mediated by different genetic factors (Hamblin and White 2000). Aflatoxin resistance could result from expression of host genes that interfere with the aflatoxin biosynthetic pathway. Nevertheless, breeding efforts to improve aflatoxin resistance in maize should incorporate aflatoxin quantification as a tool when selecting resistant germplasm.

List of several breeding and germplasm lines with improved resistance to aflatoxin in maize is given in Table 11.7. The diverse genotypes such as Mp715, Mp313E, and Mp717 from the tropical maize race Tuxpeno, Tx739, Tx736, Tx740, and Tx772 from Argentina and Bolivia, and line GT603 from temperate elite cultivars have erstwhile been used in genetic mapping studies to find the quantitative trait loci (QTL) accountable for conferring aflatoxin resistance.

Majority of the aflatoxin-resistant lines identified have tropical germplasm in their background that have undesirable agronomic qualities such as low yielding, tall, late and are prone to lodging. Due to the polygenic nature of host resistance to aflatoxin, it has been difficult to transfer the resistance from these older breeding lines into more agronomically suitable cultivars using only phenotypic selection. However, few newest breeding lines including Mp718, Mp719, Tx736, Tx739, and Tx740 show better plant type and high resistance. Another line KO679Y is 25% shorter compared to other currently available inbred lines (Henry et al. 2012). Genetic enhancement of maize (GEM) program has identified some promising accessions for aflatoxin resistance, and their further characterization is under progress (Li et al. 2002; Henry et al. 2013). An aflatoxin association-mapping panel comprising 300 maize lines has been publicly released, of which 30–40 lines showing good resistance in seven environments are accessible (Warburton et al. 2013).

To develop lines capable of sustaining resistance to aflatoxin formation over time and in different environments, it is essential to pyramid diverse resistance traits in one genetic background. Efforts are on-going to identify QTLs linked with aflatoxin resistance. To incorporate aflatoxin resistance in elite cultivars, both marker-assisted selection and traditional breeding methods could be used (Williams et al. 2003).

Table 11.7 Source of aflatoxin-resistant germplasm in maize

Resistant genotype	Screening technique	Location	References
Mp313E	Pinbar and other inoculation techniques	USDA-ARS at Mississippi State University	Scott and Zummo (1990a, b)
Mp420	Pinbar and other inoculation techniques	USDA-ARS at Mississippi State University	Scott and Zummo (1992)
GT-MAS: gk (reg. no. GP-241, PI561859)	Under both field and laboratory screening	USDA-ARS and Georgia Experimental station	McMillian et al. (1993)
T115	Field screening	Mississippi State University	Campbell and White (1995)
Mp715 (Reg. no. GP-362, PI 614819)	Field screening	USDA-ARS at Mississippi State University	Williams and Windham (2001)
Mp717 (Reg. no. GP-456, PI 639919)	Field screening	USDA-ARS at Mississippi State University	Williams and Windham (2006)
GT601 (AM-1) and GT602 (AM-2)	Field screening	USDA-ARS and Georgia Experimental station	Guo et al. (2007)
TZAR101, TZAR102, TZAR103, TZAR104, TZAR105, and TZAR106	Both field and kernel screening assay	International Institute of Tropical Agriculture and Southern Regional Research Center of the USDA-ARS.	Menkir et al. (2008)
Mp317	Field screening	USDA-ARS at Mississippi State University	Henry et al. (2009)
Tx736, Tx739, Tx740	Field screening	Texas A&M	Mayfield et al. (2012)
GT603	Under both field and laboratory screening	USDA-ARS and Georgia Experimental station	Guo et al. (2011)
Mp718 and Mp719	Field screening	USDA-ARS at Mississippi State University	Williams and Windham (2012)
KO679Y and Cuba117: S15-101-001-B-B-B-B	Field screening	USDA-ARS at Mississippi State University	Henry et al. (2012)
MI82	Kernel screening assay	USDA-ARS at Mississippi State University	Rajasekaran et al. (2013)
TZAR101, TZAR102	Field screening	USDA-ARS at Mississippi State University	Brown et al. (2016)

11.7.1.3 Cotton

Cottonseeds have three main barriers against fungal infection: the waxy layer of the seed coat, seed coat integrity, and innate immunity (Halloin and Leigh 1983). To date, no extensive evaluation of cotton cultivars for resistance to *A. flavus* infection and aflatoxin contamination has been reported.

11.7.2 Aflatoxin Detection Methods

Food safety concerns give rise to development of various aflatoxin detection and quantification techniques. Among them, widely used chromatographic methods such as thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are most accurate but expensive, while spectroscopic methods such as liquid chromatography mass spectroscopy (LCMS) have level of detection higher than 4 ppb. Immunological methods such as enzyme-linked immunosorbent assay (ELISA) are simple, specific, and suitable for onsite and routine analysis, while recently use of immunosensors allows label-free, sensitive, rapid, and accurate quantification of aflatoxin (Wacoo et al. 2014). Some recent reviews summarize the current analytical approaches for aflatoxin detection and the modern research in rapid and non-invasive detection methods (Yao et al. 2015; Berthiller et al. 2018).

11.7.3 Inheritance of Resistance

Inheritance studies are critical to reveal the nature and magnitude of gene action controlling resistance that aids in the development of appropriate breeding procedures. In maize, various reports indicated the importance of additive genetic effects (GCA) than dominance or epistatic effects (SCA) for aflatoxin resistance through Diallele and Generation Mean analysis (Zuber et al. 1978; Widstrom et al. 1984; Darrah et al. 1987; Hamblin and White 2000; Williams et al. 2018). In contrast, few reports showed that SCA was highly significant and accounts for most of the genetic variation (Gardner et al. 1987; Gorman et al. 1992; Campbell and White 1995; Walker and White 2001; Busboom and White 2004). Other reports indicate the importance of both additive and non-additive genetic variance for aflatoxin resistance (Cambell et al. 1997). This deviation could have been due to variability in inadequate scale to measure *A. flavus*, inoculation technique, non-allelic gene interaction, failure to meet assumptions (presence of significant difference in resistant parents) made in generation mean analysis. Resistance to aflatoxin contamination in corn showed variable heritability estimates, 1–29% by Busboom and White (2004), 63% by Hamblin and White (2000), and 58% by Maupin et al. (2003), depending on the environment and genotype studied. Low heritability estimates make it challenging to select for those

traits of interest and limit corn breeders to use marker-assisted selection as the only valid mean of selecting for resistance to these traits.

In peanut, the genetics of inheritance mechanism for resistance to aflatoxin is not clearly established. Nonetheless, few reports provide information on additive and dominant or epistatic gene action and heritability estimate (low to moderate) of resistance sources (Upadhyaya et al. 1997; Xue et al. 2004a, b; Arunyanark et al. 2010; Girdthai et al. 2010). No significant correlation among all the three components of resistance, pre-harvest, post-harvest, and aflatoxin production suggest that they are inherited independently (Utomo et al. 1990; Upadhyaya et al. 2002). Various studies reported significant relationship between physiological traits for drought resistance such as HI (harvest index), SLA (Specific leaf area) and SCMR (SPAD chlorophyll meter reading), and aflatoxin contamination (Arunyanark et al. 2010; Girdthai et al. 2010). Hence, these drought resistance traits can be used as indirect selection tools for aflatoxin resistance. Further research on inheritance studies requires more precise techniques to determine allelic relationship among resistance components that help plant breeders to screen suitable parents for hybridization and incorporate high level of resistance into elite cultivars.

11.7.4 Simulation Models to Predict Aflatoxin Risk

Simulation models could be used as prediction tool for managing aflatoxin in risk-prone areas. Several field and in vitro simulation models have been developed based on the relationship of interaction that occur among the fungus, temperature, and water activity (Chauhan et al. 2016). Major challenges to model aflatoxin contamination are lack of correlation between in vitro and in vivo experiments and interactions among many factors such as crop, climate, and soil (Payne et al. 1986; Probst and Cotty 2012). To evaluate aflatoxin risk in maize and peanut grown in Australia, simulation model integrated with the Agricultural Production Systems Simulator (APSIM) has been developed (Chauhan et al. 2008, 2010). To determine aflatoxin risk index (ARI), soil water availability during crucial pod filling in peanut, soil moisture, and seasonal temperature during critical silking period in maize were used. More recently, in order to predict the risk of aflatoxin contamination in maize from growing season to harvest, an automatic weather-driven model was established based on the *A. flavus* infection cycle (Battilani et al. 2013). While these models have tremendous applications, a major limiting factor in developing countries is the reliance on computerized systems and network connectivity. In future, use of smart phones could effectively overcome this limitation. The far-reaching use of mobile phones is introduced at this time to revolutionize the livestock sector in Kenya (www.fao.org/news/story/en/item/170807/icode/).

11.8 Molecular Breeding Approaches

Various molecular biology tools including genetic markers, marker-assisted selection, microarrays, transcriptomics, proteomics, genomewide association studies, and RNA-sequencing have been used as tools to develop varieties resistant to several traits of economic interest. Such strategies briefly described in the following sub-sections facilitate rapid introgression and pyramiding of aflatoxin resistance traits into susceptible varieties for more enduring aflatoxin resistance through utilizing simple, cost-effective, and high-throughput sequencing technologies that can complement traditional methods of plant breeding (Bhatnagar-Mathur et al. 2015; Bhatnagar et al. 2018; Hawkins et al. 2018; Ojiambo et al. 2018).

11.8.1 Genomewide Association Mapping

Genomewide association (GWAS) is a complementary approach to QTL mapping analysis that allows fine resolution to find association between specific loci and traits such as aflatoxin resistance using single nucleotide polymorphisms (SNPs). While mapping of quantitative trait loci (QTLs) is limited to genetic diversity present in the parents used in a bi-parental cross, nested association mapping (NAM) and multi-parent advanced generation intercross (MAGIC) populations were used in GWAS which relies on historical recombination events occurring within natural populations to identify markers linked to trait of interest can overcome the limitations of linkage analysis.

In maize, genotype by sequencing-GWAS approach has been used to identify single SNPs associated with aflatoxin resistance (Warburton et al. 2013). Identified SNPs were further validated using metabolic pathway analysis that study the cumulative effects of multiple genes classified based on their common biological function (Tang et al. 2015). Further, there is a strong correlation between the upregulation of genes linked to jasmonic acid (JA) biosynthetic pathway and lower aflatoxin content in seeds (Tang et al. 2015). Apart from JA pathway genes, genes from other pathways such as leucine-rich repeat protein kinase, expansin B3, reversion-to-ethylene sensitivity, and an adaptor protein complex gene were highly expressed in the aflatoxin-resistant lines. Combining GWAS data with pathway analysis is a robust approach to explore the genetic basis of a trait (Wang et al. 2007; Tang et al. 2015).

A GWAS study in maize utilizing 60,000 SNPs reported ten quantitative trait variants (QTVs) for seed yield, plant and ear height, days to anthesis and days to silk with certain overlapping regions to earlier identified linkage QTL, while others were novel, thereby signifying the effectiveness of GWAS to resolve and find valuable variations (Farfan et al. 2015). However, this study as well failed to find any significant QTL for aflatoxin resistance. Another study employing GWAS identified 25 SNPs linked to aflatoxin resistance, with majority of them co-localized with qAA8 (highly significant QTL that affected aflatoxin accumulation) and contributes 6.7–26.8% of

the observed phenotypic variation. Based on the linkage disequilibrium (LD), GWAS method further localized qAA8 to a short genomic region of nearly 1500 bp. Thus, qAA8 region will be valuable for a marker-assisted selection (MAS) of *A. flavus* resistance and a characterization of the fundamental gene (Zhang et al. 2016).

11.8.2 Trait Mapping and Marker-Assisted Selection

The DNA markers or QTLs linked to aflatoxin resistance gene may be used as molecular tools for MAS to interrogate resistance genes into improved varieties (Brown et al. 2013). Further, pyramiding resistant QTLs using MAS provides opportunities to develop aflatoxin-resistant lines. Although, the cultivated varieties of peanut showed large phenotypic variability in agronomic and morphological traits, genotypic molecular marker studies have identified little variation in aflatoxin resistance (Clavel 2000). Earlier efforts to link seed storage protein markers displaying varied electrophoretic profiles with aflatoxin resistance have been ineffective (Clavel 2000). To improve the reliability of resistance marker, the amplified fragment length polymorphism (AFLP) markers linked to peanut seed infection was converted into stable sequence-characterized amplified region (SCAR) marker named as 'AFs-412' (Lei et al. 2006). DNA markers for aflatoxin resistance have also been identified from an interspecific population derived from a cross between *Arachis hypogaea* and *A. cardenasii* (Rowe 2009). Due to limited genetic diversity in the cultivated peanut, genetic maps constructed so far could not go above 200 markers that are not very satisfactory, considering the large genome size, allotetraploid nature, and 20 linkage groups (Stalker and Mozingo 2001).

Due to their abundance, simple sequence repeats (SSR) are the most desirable molecular markers for genomic studies in cultivated peanut hyper-variability and suitability for high-throughput analysis. Guo et al. (2013) developed over 15,518 SSR markers during 2002–2012 from EST sequences. Kanyika et al. (2015) identified 139 informative SSR markers associated with resistance to certain peanut diseases (early leaf spot, rosette disease and rust), and aflatoxin content (AC) that have been mapped to the *Arachis* genome and can be employed in QTL mapping. In *Arachis cardenasii* derived lines, a set of six AFLP markers with low phenotypic variance explained (PVE) was identified (Milla et al. 2005). While six QTLs for resistance to *A. flavus* infection with PVE ranging from 6.2 to 22.7% were identified (Liang et al. 2009), it has been asserted that crop's resistance to *A. flavus* colonization and AC should be quantitative, and the resistance is severely influenced by environmental interactions (Fountain et al. 2015). Consequently, identifying consistent QTLs for resistance to AC has been a difficult task since breeding efforts to discover and characterize QTLs for resistance to AC were forced to consider the environment in obtaining phenotypic data (Fountain et al. 2015).

In recombinant inbred line (RIL) population derived from cross between Tifrunner × GT-C20, 16 QTLs were identified of which five QTLs were associated with aflatoxin contamination and eleven with fungal growth (Ji et al. 2016). Among these,

five major QTLs explained more than 10% of the total PVE. Of five major QTLs, one QTL linked with aflatoxin contamination accounted for 10.14% PVE, and three QTLs linked with fungal growth accounted for 13.23, 11.21 and 14.17% PVE, respectively. Further, a comparison of these QTLs with the recently released peanut diploid genome were planned to identify putative resistance genes and their validation for potential applications in breeding.

Advances in molecular plant breeding in the past decade have led to the development of aflatoxin-resistant and agronomically acceptable maize varieties. Several molecular markers that were linked to QTLs, like microsatellite markers, RFLPs, insertion–deletion (InDel) markers, and SNP markers are being used in mapping efforts (Williams et al. 2015). With the development of statistical methods, recently multiple QTLs were mapped together using multiple interval mapping (MIM) model for quantitative traits such as aflatoxin resistance (Willcox et al. 2013).

Several potential QTLs for aflatoxin resistance and *Aspergillus* in maize were identified through mapping and GWAS (Brooks et al. 2005; Bello 2007; Warburton et al. 2009, 2011a, b; Mayfield et al. 2011; Willcox et al. 2013; Yin et al. 2014), and few of them have been identified in multiple studies and/or from multiple donor lines (Table 11.8). QTLs stable over different genetic backgrounds or varied environmental conditions are useful in MAS. RFLP analysis in three 'resistant' lines reveals that QTLs linked to resistance to *Aspergillus* ear rot and aflatoxin accumulation were located on separate chromosomal regions (White et al. 1998). QTLs linked to aflatoxin resistance have been mapped to the chromosome regions such as the glycine-rich RNA binding protein (GRB2) at bin 1.06, heat shock protein 18a (HSP18a) at bin 9.05, heat shock protein 26 (HSP26) at bin 1.03, the NPCs-NUP85-RNA transport protein at bin 5.05, and lecithin cholesterol acyltransferase-like protein (LCAT) at bin 2.06 (Kelley et al. 2012). Furthermore, certain maize resistance-associated proteins (RAPs) such as heat shock protein 17.2 (HSP17.2)/Glyoxylase (GLX) at bin 1.03, trypsin inhibitor (TI) at bin 2.06, a glucose dehydrogenase at bin 2.08, a glucanase at bin 3.05, and an embryo-specific protein at bin 4.06, respectively, have been mapped through sequence homology analysis (Chen et al. 2012). QTLs identified in this study co-locate with other QTLs identified in previous studies such as bin 2.06 and 4.06 (Brooks et al. 2005), bin 3.05 and 3.06 (Paul et al. 2003), bin 3.06 and 4.06 (Warburton et al. 2011a, b), and bin 2.08 (Busboom and White 2004). These data indicate the potential role of RAPs in imparting aflatoxin resistance.

In a mapping population derived from CML161 × B73o2 cross, only five of 38 epistatic interactions identified for reduced aflatoxin had one locus with significant main effect (Bello 2007), thereby indicating that the epistatic effect is not consistent across different locations. This genotype by environment interaction has also been reported in other studies (Paul et al. 2003; Brooks et al. 2005; Warburton et al. 2009). Hence, QTL studies for quantitative traits such as aflatoxin need to be tested in various locations to minimize errors in heritability and QTL estimates. In addition, evaluation of QTLs across different genotypes and hybrids or RIL populations as testcross provides better estimation of QTL than the lines perse. Variations for the other secondary traits such as harder kernel texture and longer husk coverage that are correlated with aflatoxin resistance have also been genetically mapped besides

Table 11.8 Summary of QTLs associated with aflatoxin resistance in maize

Parents	Population type	Population size	Approach	No. of Markers	Chromosome	Phenotypic variance	References
Tex6 × B73	F2:3	100	CIM MR	SSR and RFLP	3, 4, 10	6.7–15.1	Paul et al. (2003)
Tex6 × B73	BC1S1	176	CIM MR	SSR and RFLP	5	16.1–17.8	Paul et al. (2003)
B73 × Oh516	BC1S1	217		SNP	2, 3, 7		Busboom and White (2004)
Mp313E × B73	F2:3	210	CIM	SSR	1, 2, 3, 4, 5, 6	5–45.7%	Brooks et al. (2005)
Mp717 × NC300	F2:3	270	CIM	SSR	1, 2, 3, 5, 7, 8, 10	1–11%	Warburton et al. (2009)
Mp715 × T173	F2:3	225	CIM	SSR and one gene-based marker	1, 3, 4, 5, 9, 10	2.7–18.5%	Warburton et al. (2011a, b)

(continued)

Table 11.8 (continued)

Parents	Population type	Population size	Approach	No. of Markers	Chromosome	Phenotypic variance	References
Mp313E × Va35	F2:3	216	CIM, MIM	RFLP and SSR	1, 2, 3, 4, 5, 6, 7, 8, 9	0–21%	Willcox et al. (2013)
B73 × CML322	F2S5	185	Stepwise regression and CIM	SNP	4, 7, 8, 9, 10 by stepwise regression approach and all chromosome except 9 by CIM model	21–41%	Mideros et al. (2014)
(B7302/02 × CML616) × LH195	Test cross	146	Single marker analysis, Interval mapping, and CIM		1, 3, 4, 8, 9	7–26%	Mayfield et al. (2011)
RA × M53	F7:8	228	CIM and MCIM	SSR and SNP	5, 6, 8, 10—CIM, 1, 2, 5, 6-MCIM	3.55–9.87%	Yin et al. (2014)
B73 × Mp715	F2:3	210	CIM	SSR	2, 3, 4, 5, 8, 9, 10	<1–10%	Dhakal et al. (2016)

Proportion of phenotypic variance explained by QTL, *SSR* Simple Sequence Repeat, *CIM* Composit Interval Mapping, *SNP* Single Nucleotide Polymorphism markers, *MCIM* Mixed model based on CIM

maturity, endosperm texture, percentage of rotten ears, and grain yield per ear (Bello 2007). Mapping component of resistance to *A. flavus* would uncover novel QTLs compared to previous studies that mapped resistance to aflatoxin accumulation itself. The identification of these traits can help in indirect selection for aflatoxin resistance in marker-assisted breeding.

A major QTL (*p1*) has been reported in bin 1.03 at the pi locus for silk antibiotic compounds that contributes 54.0, 42.1, and 28.3% of the phenotypic variability for silk *mays*in, 3′-methoxy*mays*in/api*mays*in, and chlorogenic acid concentrations, respectively (Widstrom et al. 2003). In addition, QTLs for husk tightness were also detected on chromosome 1S, IL, 3L, and 7L and for total aflatoxin concentrations on chromosome 2L and 1S. The p1 locus located on chromosome 1 has been repeatedly overlapped with a major QTL affecting *mays*in production (Zhang et al. 2003; Mayfield et al. 2011). MpM1, Mississippi marker 1 is the first gene-based marker that has been successfully used in MAS for transferring aflatoxin resistance in maize (Mylroie et al. 2013). This provides evidence that while complicated it is feasible to achieve improvement in molecular breeding approaches for aflatoxin resistance and *Aspergillus* ear rot (Williams et al. 2003; Brooks et al. 2005). To unravel the genetic and molecular mechanism involved in aflatoxin resistance, markers tightly linked to the QTL, preferably at <5 cm genetic distance were required that expedite the breeding process by reducing the cycle time (Shan and Williams 2014). To identify consensus QTL for aflatoxin resistance across studies, meta-analysis of *A. flavus*, aflatoxin, and ear rot resistance was carried out using all the available data sets in maize through multiple QTL mapping populations (Warburton et al. 2011a, b; Mideros et al. 2014), where 12 independent QTLs, seven in bins 4.07–4.08, and five in bin 4.09 were detected. The bin 4.08 have the largest effect QTL having over twofold the predictable number of QTLs for many diseases suggesting that this region has a group of genes manipulating the response to various pathogens (Mideros et al. 2014). Further the stability of these larger effect QTL regions was verified across different varieties through backcrossing (Mideros et al. 2014). Similarly, QTL mapping produced more robust results for field evaluated traits than for in vitro traits (Mideros et al. 2012). Main aflatoxin reducing QTL which performed stably across various locations was identified in bin 5.03 and seven other QTL were detected in single environment (Yin et al. 2014). Moreover, a chitinaseA located in bin 2.04 was linked with a large aflatoxin reducing QTL, as described by Hawkins et al. (2015). Further, QTL regions were found for aflatoxin resistance on chromosome 2, 4, 5, and 10 that were validated by gene expression of pathogenesis-related protein 4, leucine-rich repeat (LBR) family protein, and DEAD-box RNA helicases in resistant inbred lines through real-time PCR analysis (Dhakal et al. 2017). *In*-silico mapping showed coinciding of many genes implicated in stress response, disease resistance, and metabolism within the QTL regions.

Evaluating the QTL identified from Mp313E in a background of the susceptible inbred line Va35, 20 QTL were identified explaining 22–43% of phenotypic variation within a F2 mapping population derived from Mp313E × Va35 (Willcox et al. 2013). Among the 20 identified QTLs, 11 were consistently expressed over various locations that accounted for 2.4–9.5% of phenotypic variance. By comparing this QTL with

those previously reported, five QTL found in bins 1.02, 2.05, 3.05, 4.06, and 5.01 seemed to be the same as those identified in Mp313E × B73. Stable QTLs identified in this study will be valuable in breeding efforts to develop aflatoxin-resistant maize lines. Brooks et al. (2005) analyzed the phenotypic data from three locations where it was possible to identify two consistent QTLs, one with PVE of 7–18% and the second with PVE of 8–18%, thereby suggesting a lower variation across various locations. A single consistent QTL (PVE 8.42%) was identified in a RIL population derived from RA × M53 (resistant × susceptible) utilizing 916 SNP markers in two locations (Yin et al. 2014). However, QTLs identified in most of the studies reported multiple QTLs that were detected in only a single environment, where most contributed less than 5% of the phenotypic variation observed in the population and the environment in which they were measured (Warburton and Williams 2014).

11.8.3 Identification of Candidate Gene Through 'Omics' Approaches

In recent years, *A. flavus*-host interactions have been studied by various omics approaches such as genomics, transcriptomics, and proteomics, etc. ESTs generated from cDNA fragments and microarray technology provides a genomewide gene expression, and thereby associate genes with predictive functions or specific physiological conditions.

Transcriptome analysis of maize, peanut, and cotton during infection with *A. flavus* and drought stress has provided insights into the mechanisms underlying aflatoxin resistance and identify genes related to aflatoxin resistance. Recently, the advent of high-throughput sequencing has facilitated studies on EST and microarrays have led to the identification of key gene networks that respond to aflatoxin stress and relating their regulation to various developments manifested during stress (Wang et al. 2013; Dolezal et al. 2014). Similarly, gene expression profiling of maize kernels during *A. flavus* infection has been studied using cDNA and oligo microarrays (Luo et al. 2008, 2010, 2011; Wilkinson et al. 2007; Kelley et al. 2012) and qPCR (Jiang et al. 2011; Lanubile et al. 2017). Maize ESTs have been derived from kernels collected at different stages of development 15–45 days after pollination (DAP) and *Aspergillus* and drought stresses (Hamblin and White 2000; Dowd and White 2002; Moore et al. 2004; Guo et al. 2007). Maize cDNA and oligonucleotide arrays have also been made available through the Maize Gene Discovery Project and Maize Oligonucleotide Array Project (MOAP) (http://www.maiZearray.org).

Comparison of the gene expression profile of resistant and susceptible maize varieties in response to *A. flavus* revealed the detection of higher number of maize genes in susceptible kernels compared with resistant ones (Luo et al. 2008, 2011). Over 163 genes were found to express differentially, of which 75 genes were defense-related and remaining 88 genes were genotype-specific. All these studies indicated significant expression of defense-related genes, particularly PR genes, PR-4,

chitinases, beta-1,3-glucanases, catalase 3, *Zea*matin-like protein, peroxidases, and trypsin inhibitors. Other parallel microarray studies revealed upregulation of 123 genes involved in several metabolic pathways in susceptible maize line Va35 upon *A. flavus* infection, whereas resistant line Mp313E showed upregulation of 95 genes involved in amino acid derivative metabolism and lipid metabolism (Wilkinson et al. 2007). Subsequently, 31 highly expressed maize transcripts were reported using combination of microarray and qRT-PCR analysis and mapped to previously identified quantitative trait locus (QTL) regions (Kelley et al. 2012).

Similarly, peanut cDNA microarray revealed 52 genes to be upregulated under drought stress and 42 upregulated under both *A. flavus* and drought stress, where 25 genes were commonly expressed under both the treatments (Luo et al. 2005). Subsequent gene expression profiling of GT C20 (resistant line) and Tiffrunner (susceptible line) from developing seeds at three reproductive stages (R5, R6 and R7) in response to infection with *A. parasiticus* and drought stress generated 21,777 ESTs (Guo et al. 2008). Significant upregulation of nine genes in 'GT-C20' and eight resistance-related genes in 'Tifrunner' libraries were also reported. Utilizing these and other publicly available ESTs, a high-density oligonucleotide microarray was designed for expression studies in various tissues such as pod, leaf, stem, root, and peg tissues (Payton et al. 2009). In this study, a higher expression of pod transcripts was observed that suggested the presence of different pathways involved in the generation of secondary metabolites, storage, and desiccation-related proteins. Using RT-PCR, the expression of eight differentially expressed transcripts was also validated. A subsequent report with peanut microarrays showed that 'GT-C20' (the R line) had a greater response to *Aspergillus* infection compared with 'Tifrunner' (the S line) where over 62 genes were upregulated in resistant cultivar, of which only eight genes were assigned biological functions based on their homology against annotated entries in the GenBank database (Guo et al. 2011). In a recent microarray study, 490 unigenes involved in 26 pathways were reported to be differentially expressed in the resistant genotype YJ1 which uniquely responded to *A. flavus* infection under drought stress, whereby 96 DEGs were related to eight metabolic pathways (Wang et al. 2013). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that all the eight networks were found to be significantly associated with resistance to *A. flavus* infection in resistant genotype YJ1 compared with susceptible Yueyou 7. This suggests that *A. flavus*–peanut interaction is controlled by many metabolic pathways (Guo et al. 2008; Wang et al. 2010). Subsequent genetic analysis of the identified resistance-related pathways is reportedly in progress to further characterize their possible functional roles in resistance to pre-harvested *A. flavus* infection.

Transcriptional monitoring of 9000 genes in inoculated maize kernels at four days after infection using Affymetrix GeneChip DNA array revealed higher expression of defense-related genes, signaling pathway genes, and genes encoding hydrolytic enzymes, while the starch biosynthesis genes were downregulated (Dolezal et al. 2014). Through a comparative analysis of *A. flavus*-responsive transcriptome of cotton with peanut and maize, 732 putative genes have been reported where 26 genes were commonly regulated in all the three crops which could be the potential candidates for aflatoxin resistance (Mehanathan et al. 2018). A diverse expression pattern

of 14 genes was observed in RT-PCR studies during seed germination in peanut after *A. flavus* infection (Zhang et al. 2014). According to the expression levels, these 14 genes were classified into six different groups associated with lipid metabolism, oxidative signaling, and cell wall synthesis during the counter-attack. An ordered pattern in the expression of the different categories of genes was observed in response to fungal invasion after inoculation.

Real-time PCR analysis of 18 defense genes in developing maize kernels at 96 h after inoculation with toxigenic and atoxigenic strains of *A. flavus* revealed higher upregulation of the genes that encode oxidative stress-related proteins, pathogenesis-related proteins, lipoxygenases, and transcriptional factors against atoxigenic strain (Lanubile et al. 2017). This indicates that overexpression of maize-defense-associated genes observed in response to the atoxigenic strain might have contributed to aflatoxin reduction. The information gained from the above microarray and qRT-PCR studies has led to the discovery of many critical genes related to aflatoxin resistance and the development of numerous molecular markers for drought tolerance and resistance to *Aspergillus* infection and aflatoxin contamination. The ESTs will continue to be actively sequenced to fill knowledge gaps and complement the whole genome sequence. In addition, this functional genomics research facilitates understanding defense mechanism of aflatoxin resistance in various crops at physical and biochemical levels.

11.8.3.1 Transcriptomics

The recently released genome sequence of *A. duranensis* and *A. ipaensis* (Bertioli et al. 2016) provided a new strategy for peanut transcriptome sequencing with better coverage and accuracy. RNA-sequencing is a rapid and high-throughput genomewide gene expression analysis which has been used to survey sequence variations and complex transcriptomes with less false-positive rates with high repeatability and accuracy. Furthermore, RNA-seq produces absolute rather than relative gene expression measurements, thereby providing deeper insights and higher sensitivity than microarrays (Zhao et al. 2018). Recently, RNA-seq approach was applied to identify transcripts that were differentially expressed in *A. flavus* interacting with the resistant vs. the susceptible peanut seed (Wang et al. 2016a, b; Nayak et al. 2017).

RNA-sequencing was used to analyze the transcriptomic profiles of *A. flavus* treated with resveratrol (Wang et al. 2015). In total, 366 and 87 genes of *A. flavus* were significantly up- and downregulated, respectively, and when the fungus underwent exposure to resveratrol, a polyphenol is isolated from red wine. Resveratrol improved the activity of enzymatic antioxidative defense system that consequently damaged free radicals, thereby leading to reduced aflatoxin production. Genes involved in primary and secondary metabolism in *A. flavus* were also affected, thereby reducing aflatoxin production and causing abnormality in fungal development and reproduction (Wang et al. 2015).

Over 4445 differentially expressed genes (DEGs) comprising resveratrol synthase, defense-related genes like senescence-associated proteins, pathogenesis-related

proteins, and 9s-lipoxygenase were identified using RNA-sequencing (Nayak et al. 2017). In *A. flavus*, around 578 DEGs that regulate pathways for growth and development of fungus, binding, transport, aflatoxin biosynthesis, and signaling were detected in compatible interactions. In addition to finding potential genes responsible for IVSC resistance in peanut, the study found the genes implicated in host–pathogen interaction and identifies the markers that can be used in breeding resistant varieties. Wang et al. (2016a, b) detected DEGs linked to mycelial growth, conidial development and aflatoxin biosynthesis that were upregulated in aflatoxin susceptible peanut compared with aflatoxin-resistant peanut, providing evidence that *A. flavus* mycelia enter readily and produce far more aflatoxin in susceptible than in resistant peanut.

Changes in expression levels of a cluster of genes in contaminated against uncontaminated peanut seed were studied by RNA-sequencing (Clevenger et al. 2016). Here, the contaminated seeds showed changes in abscisic acid (ABA) signaling and fatty acid biosynthesis and detected key susceptibility factor *ABR1* as a repressor of ABA signaling that may be involved in post-harvest aflatoxin accumulation (PAC). Recently, RNA-sequencing has also been used in cotton and maize to understand the basis of aflatoxin resistance at the molecular level by identifying and characterizing genes involved in various metabolic pathways that lead to aflatoxin contamination. In cotton, inoculated bolls harvested over different time course were investigated using RNA-sequencing where the genomewide multiple time course transcriptome analysis revealed an overexpression of genes involved in regulation of transcription factors, defense-related genes, genes involved in oxidative brust, and genes involved in synthesis of antifungal compounds and apostasis in pericarp and seeds in response to *Aspergillus* (Bedre et al. 2015).

Resistant maize lines showing higher expression of genes related to Jasmonic acid (JA) pathway, ethylene (ET) signaling pathways, and shikimate biosynthesis pathway were detected using RNA-sequence approach (Lanubile et al. 2014). Further, the maize kernels resistant to *A. flavus* and other two *Fusarium* spp., *F. proliferatum,* and *F. subglutinans* showed higher gene expression. In addition, the expression of *PR* genes remained higher in the resistant lines before and after inoculation (Lanubile et al. 2015).

11.8.3.2 Proteomics

Aflatoxin resistance is a complex polygenic and quantitatively inherited trait, controlled by several genetic loci, and substantially altered by the environment. Gene expression analysis of resistant and susceptible lines under *A. flavus* infection identified various genes involved in host plant responses using a combination of SDS-PAGE and western blots. However, due to the inconsistent band resolutions of SDS-PAGE gels, high-throughput proteomics approaches are currently being utilized as a novel tool in aflatoxin research to identify the resistance-associated proteins (RAPs) associated with aflatoxin resistance (Razzazi-Fazeli et al. 2011). Several storage and stress-related proteins have been identified that can be used as markers for aflatoxin resistance, thereby possibly being useful for breeders to design proper strategies to

develop plant resistance against *A. flavus* infestation and aflatoxin contamination. Several studies demonstrated the role of maize kernel, embryo, endosperm, silk, and rachis defense proteins in *A. flavus* infection and aflatoxin contamination. These studies indicate that major aflatoxin resistance factors were governed by constitutive proteins, although the inducible defense proteins also play an essential role. The list of various differentially expressed proteins identified by comparing resistant and susceptible genotypes is given in Table 11.9.

Mainly three categories of proteins viz., pathogenesis-related, storage proteins, and stress-responsive proteins were found to significantly increase in resistant genotypes (Pechanova and Pechan 2015). Other studies indicate the role of phenolic compounds such as alkylresorcinol to be responsible for maize kernel pericarp wax and catalase-specific activity in suppression of *A. flavus* infection/aflatoxin production (Gembeh et al. 2001; Magbanua et al. 2007).

Although several proteins have been identified, only few, such as the 14 kDa trypsin inhibitor protein (TI) has been well-characterized that was constitutively expressed at higher levels in the resistant lines, whereas low or absent in susceptible lines (Chen et al. 1998). In addition, it also displayed antifungal activities against a broad range of fungal pathogens (Chen et al. 1999a, b). Expression of the gene encoding the 14-kDa trypsin inhibitor that inhibits fungal amylases was highly expressed in kernel tissues of resistant maize lines compared to susceptible lines under *A. flavus*-drought stress (Fountain et al. 2010). The role of TI in aflatoxin resistance was further confirmed by RNAi silencing and genetic mapping studies (Chen et al. 2016). In this study, the T_1 transgenics showed significant transcript reduction of 63–88% corresponding to 39–85% reduction at protein level. Silenced transgenics with lack of TI showed increased aflatoxin contamination. These data were also confirmed by QTL mapping studies where three QTLs with log of the odds scores of 11, 4.5, and 3.0 were found to have a possible association with TI gene, and thereby aflatoxin resistance. Recent study by Gilbert et al. (2018) showed that RNAi silencing of amylase gene suppresses aflatoxin production, thereby confirming that higher expression of TI in resistant lines might inhibit amylase that hydrolyzes starch into sugar, resulting in reduced fungal growth and aflatoxin production.

Apart from maize kernels, *A. flavus* also infects rachis and silk tissues that have also been evaluated for potential protein profile differences between resistant and susceptible lines. Comparison of maize rachis proteome in resistant and susceptible lines with or without *A. flavus* infection revealed that resistant lines contained greater levels of abiotic stress-related proteins and proteins from phenylpropanoid metabolism, while those from susceptible lines contained more abundant pathogenesis-related proteins (Pechanova et al. 2011). In another study, three chitinases (PRm3 chitinase, chitinase I, and chitinase A) were found to be differentially expressed and possessed higher chitinase activity in the silk tissue of resistant maize inbred lines (Mp313E and Mp420) compared to susceptible inbred lines (SC212m and Mp339) (Peethambaran et al. 2010), suggesting that these proteins may contribute to *A. flavus* resistance. This study also suggested that the resistant lines depend on constitutive defenses, whereas the susceptible lines are more dependent on inducible defenses. Transcriptomics and proteomics studies in maize response to the *A. flavus* and *Fusarium* spp. reveal that

Table 11.9 Summary of proteins identified in maize

Proteins group	Differentially expressed protein	Tissue	References
Pathogenesis-related proteins	Chitinase	Kernel, rachis, silk	Chen et al. (2001), Ji et al. (2000), Pechanova et al. (2011), Moore et al. (2004), Peethambaran et al. (2010)
	Glucanase	Kernel, endosperm	Lozovaya et al. (1998), Ji et al. (2000), Liang et al. (2005), Chen et al. (2012)
	22 kDa alpha-amylase	Kernel, endosperm	Chen et al. (2012)
	14 kDa trypsin inhibitor	Kernel, endosperm	Chen et al. (1998, 2012), Fountain et al. (2010)
	Zeamatin	Kernel, endosperm	Chen et al. (2001, 2012)
	Thaumatin-like proteins	Kernel	Huang et al. (1997)

(continued)

Table 11.9 (continued)

Proteins group	Differentially expressed protein	Tissue	References
	Ribosome-inactivating proteins (RIP)	Kernel	Guo et al. (1997), Chen et al. (2001)
	PR10	Kernel, endosperm	Chen et al. (2006, 2007)
Detoxifying enzymes	Superoxide dismutase	Endosperm, rachis	Pechanova et al. (2011), Chen et al. (2012)
	Catalase 3	Rachis	Pechanova et al. (2011)
	Per1 peroxidinin	Endosperm	Chen et al. (2012)
	Thioredoxin-related redox proteins	Rachis	Pechanova et al. (2011)
	Dehydroascorbate reductase	Rachis	Pechanova et al. (2011)
	APx1-cytosolic ascorbate peroxidase	Rachis	Pechanova et al. (2011)
	Hydroxyacylglutathione hydrolase	Rachis	Pechanova et al. (2011)

(continued)

Table 11.9 (continued)

Proteins group	Differentially expressed protein	Tissue	References
Proteins involved in secondary metabolism or phenylpropanoid metabolism	Caffeoyl-CoA 3-*O*-methyltransferase	Rachis	Pechanova et al. (2011)
	Chalcone-flavonone isomerase	Rachis	Pechanova et al. (2011)
Late embryogenesis abundant proteins	LEA3	Endosperm	Chen et al. (2012)
	LEA14	Kernel	Chen et al. (2002, 2007)
Storage proteins	Globulin 1	Kernel	Chen et al. (2002)
	Globulin 2	Kernel, embryo, endosperm	Chen et al. (2002, 2012)

(continued)

Table 11.9 (continued)

Proteins group	Differentially expressed protein	Tissue	References
	Cupin-domain-containing protein (Zmcup)	Embryo	Chen et al. (2012)
	Vicillin	Embryo	Chen et al. (2012)
Stress-responsive proteins	Heat shock proteins	Kernel, rachis, embryo	Pechanova et al. (2011), Chen et al. (2002, 2007, 2012)
	Chaperonin	Rachis	Pechanova et al. (2011)
	Peptidyl-prolyl isomerase	Rachis	Pechanova et al. (2011)
	Aldose reductase	Kernel	Chen et al. (2002)
	Glyoxalase I (Glx I)	Embryo	Chen et al. (2012)
	Peroxredoxin (Per1)	Kernel	Chen et al. (2007)
	Cold-regulated protein	Embryo	Chen et al. (2012)
	Water stress inducible protein	Kernel	Chen et al. (2002)
	Anionic peroxidase	Kernel	Chen et al. (2007)
	ABA responsive protein	Rachis	Pechanova et al. (2011)
	Cysteine proteinase inhibitor 2	Rachis	Pechanova et al. (2011)
	Acyl-CoA-binding protein	Rachis	Pechanova et al. (2011)
	Triose phosphate isomerase	Rachis	Pechanova et al. (2011)
	Proteasome subunit b	Rachis	Pechanova et al. (2011)
Other cellular process	Eukaryotic translation initiation factor 5A	Rachis, endosperm	Pechanova et al. (2011), Chen et al. (2012)
	Adenosine kinase 2	Rachis	Pechanova et al. (2011)
	Putative lipid transfer protein	Endosperm	Chen et al. (2012)

several stress proteins such as PR proteins, detoxifying enzymes, and enzymes from the phenylpropanoid pathway were commonly overexpressed under infection with either fungus (Mohammadi et al. 2011; Pechanova et al. 2011). To make the data on the reported maize RAPs available and to promote its usage, Kelley et al. (2010) constructed a relational database with web interface to integrate results from available data sets (microarray, proteomics, QTL studies, and SNP data). There are multiple lines of evidence that show the expression of DRE binding factor 1 (AW438135), PR-1 (TC239060), and a disease resistance protein PRM1 (AZM4_24463) that were associated with maize aflatoxin resistance. These candidate genes database facilitates QTL-based candidate gene identification that helps in developing aflatoxin-resistant maize cultivars through marker-assisted selection or else using transgenic technology (Kelley et al. 2012).

More recently, 220 differentially expressed proteins (DEPs) have been identified by deploying proteomics for three different *A. flavus* isolates in response to H_2O_2-derived oxidative stress (Fountain et al. 2018). These discovered DEPs involved various metabolic pathways including antioxidants, carbohydrates, pathogenicity, and secondary metabolism. Isolate-to-isolate variation in oxidative stress tolerance reported in this study enhanced understanding of the host–plant interactions under drought stress to design more targeted efforts in host resistance research.

As compared to maize, there have been limited reports on comparative proteomics in peanut. In a study by Liang et al. (2005), resistant varieties of peanut showed three to fourfold increase in β-1,3-glucanase against *A. flavus* infection indicating that this enzyme targets inducible defenses in peanut. Comparison of seed protein profiles between a resistant and a susceptible cultivar under *A. flavus*–drought stress has identified 12 proteins with significant upregulation of signaling proteins, SAP domain-containing protein, storage proteins, stress-responsive proteins, 50 S ribosomal protein L22, and putative 30 S ribosomal S9 and significant downregulation of trypsin inhibitor (Wang et al. 2010). Using differential proteomic approach, array of proteins responding to aflatoxin in peanut cotyledons infected with aflatoxin-producing and non-producing strains has been identified. These proteins are involved in immune signaling and PAMP perception, DNA and RNA stabilization, induction of defense, innate immunity, hypersensitive response, biosynthesis of phytoalexins, cell wall responses, peptide glycan assembly, penetration resistance, condensed tannin synthesis, detoxification, and metabolic regulation (Wang et al. 2012). This study revealed that aflatoxin triggers an immune response for disease resistance in peanut cotyledons and a mechanism for detoxification and DNA repair in *A. flavus*. Gene expression in atoxigenic strain indicates that the fungal genes can respond to aflatoxin, whether the strain produces aflatoxin or not.

11.8.4 Applicability of RAPs as Breeding Markers

Various studies have demonstrated the potential contribution of identified proteins in resistance to aflatoxin (Brown et al. 2010). For instance, constitutive overexpres-

sion of aldose reductase was shown to have a role in stress tolerance (Woloshuk et al. 1997), such as pathogenesis-related proteins like *Zea*matin and RIP showed inhibition of *A. flavus* growth in vitro (Chen et al. 2001), Glyoxalase I reduced methylglyoxal (MG) that induces transcription of *aflR* (Chen et al. 2004), and ribonucleolytic activity of PR-10 demonstrated inhibition of *A. flavus* growth (Chen et al. 2010). Host-induced gene silencing of PR10 expression increased aflatoxin contamination in maize indicating a significant role of PR-10 in resistance to aflatoxin (Chen et al. 2010). While a cold-regulated protein like ZmCORp has been reported to impede mycelial growth and *A. flavus* germination (Baker et al. 2009), Cupin-domain-containing proteins in maize have been shown to act as transcription factors or enzymes in aflatoxin resistance; however, their function is not clearly understood (Dunwell et al. 2004).

Some of identified targets such as trypsin inhibitor and PR10 have already been used in RNAi-mediated resistance development (Chen et al. 2010, 2016). Some RAP genes have also been mapped to chromosome regions containing major QTL such as TI at bin 2.06, embryo-specific protein (spot 337) at bin 4.06, HSP17.2/GLX at bin 1.03, glucanase at bin 3.05, and glucose dehydrogenase at bin 2.08. These RAPS associated with major QTLs linked to aflatoxin resistance could assist in prioritization of candidate genes that should be tested as markers. However, comprehensive analysis of target proteins is required to further characterize their function at the genomewide scale.

11.9 Genetic Engineering Approaches

Genetic engineering offers numerous benefits over other traditional methods such as biological control and genetic resistance for enhancing germplasm with novel traits that may not be available in the existing germplasm. The transfer of traits controlled by single or few closely linked transgenes into elite cultivars is a much more attainable and speedy approach than the introgression of genes governing quantitative resistance due to lack of potential markers. Since achieving a high level of resistance to aflatoxin has been difficult through conventional breeding methods due to high GxE interactions, and the use of genetic engineering has become potentially important in crop like maize, peanut, and cotton (Tomovska et al. 2012). Studies on crop–fungus interactions have identified several compounds that are inhibitory to fungal growth, including trypsin and amylase inhibitors, ribosome-inactivating proteins, and chitinases. These host proteins that contribute to enhanced resistance have paved the pathway for development of aflatoxin-resistant transgenic crops. Several aflatoxin resistance genes from natural resources have been cloned and introduced into crop plants through genetic engineering. An overview of various resistance-associated proteins and genes identified through application of in vitro antifungal assays, genomics and proteomic studies, and transgenic strategies aimed at aflatoxin resistance is provided in Tables 11.10 and 11.11.

Table 11.10 Key proteins and their function in antifungal activity against *Aspergillus flavus*

Source	Protein name	Protein family	Molecular function	References
Pseudomonas pyrrocinia	Haloperoxidase	Peroxidase	Catalyze formation of antimicrobial compounds—peracetic acid and hypohalites	Jacks et al. (2000), Rajasekaran et al. (2000)
Nicotiana tabacum	β-1-3 glucanase	Glycosyl hydrolase	Hydrolysis of fungal cell wall	Ji et al. (2000), Lozovaya et al. (1998)
Ipomea batatas	Ib-AMP3	Defensin	Lytic	De Lucca (1998)
Lablab purpureus	AILp	Lectin	Inhibits germination and hyphal growth	Fakhoury and Woloshuk (2001)
Zea mays	Chitinase	Glycosyl hydrolase	Hydrolysis of fungal cell wall components	Moore et al. (2004)
Zea mays	*ZmCORp*	Lectin	Hemagglutination activity against fungal conidia	Baker et al. (2009)
Zea mays	Mod-1/RIP-1	Ribosome-inhibiting protein	Inhibits hyphal tip growth by modifying or inactivating foreign ribosome	Nielsen et al. (2001), Weissinger et al. (2007)
Zea mays	Zeamatin	PR-5	Inhibits hyphal tip growth by increasing permeability of cell membrane	Guo et al. (1997)
Zea mays	ZmPR-10	PR-10	RNAse activity	Chen et al. (2006)
Zea mays	Trypsin inhibitor	Protease inhibitor	Trypsin/amylase inhibition	Chen et al. (1998)
Hordeum vulgare and *Triticum aestivum*	Purothionin hordothionin	Thionin	Lytic	Rajasekaran unpublished
Synthetic peptide	D4E1	Synthetic peptide	Reduced *A. flavus* spore germination by 50%	Cary et al. (2000c, Rajasekaran et al. (2005)

(continued)

Table 11.10 (continued)

Source	Protein name	Protein family	Molecular function	References
Zea mays	Glyoxylase	Regulates methylglyoxal levels	Contribute to the lower levels of aflatoxins found in resistant maize genotypes	Chen et al. (2004)
Synthetic peptide	D5C/D5C1	Antimicrobial peptide	Lytic	Weissinger et al. (2000)
Synthetic peptide	D2A21	Antimicrobial peptide	Lytic	Weissinger et al. (2000)
Synthetic peptide, analog of maganin 2	MSI99	Antimicrobial peptide	Lytic—inhibit growth of pre-germinated spores of *A. flavus* by more than 95%	DeGray et al. (2001)
Spined soldier bug (*Podisus maculiventris*)	Thanatin	Antimicrobial peptide	Threefold increase in resistance to *A. flavus* infection compared with control lines	Schubert et al. (2015)
Synthetic peptide	Tachyplesin1-derived synthetic peptide AGM182	Antimicrobial peptide	Lytic	Rajasekaran et al. (2018)

AMP Antimicrobial peptides, *PR* Pathogenesis-related proteins

11.9.1 Transgenic Strategies to Reduce Both A. Flavus *Growth and Aflatoxin Contamination*

Various antifungal genes from bacterial, plant, and mammalian sources have been shown to afford protection against *A. flavus* growth and aflatoxin production in transgenic plants. Small antimicrobial lytic peptides like D4E1, D5C, and tomato anionic peroxidase (tap 1) show antifungal activity in vitro and effectively reduce the seed infection level in plants transformed using these genes (Ozias-Akins et al. 1999; Weissinger et al. 1999; Cary et al. 2011). The synthesis of these stable and target-specific peptides was aimed to prevent proteolytic degradation and non-specific toxicity to non-target organisms caused by the lytic peptides (Marcos et al. 2008). While aflatoxin production has been reported to be reduced by overexpression of synthetic analogs of cecropins and magainins (Cary et al. 2000a, b, c; DeGray et al. 2001), sequence modification has increased the potency of these peptides.

Table 11.11 List of genes encoding key enzymes/proteins used in transgene mediated aflatoxin resistance

Crop	Construct used for transformation				Performance	References
	Gene name	Gene function	Gene source	Promoter		
Tobacco	Defensin D4E1 gene	Antimicrobial peptide	Synthetic peptide	d35S	Significantly reduced in vitro fungal colonization	Cary et al. (2000c)
Tobacco	Non-heme chloroperoxidase gene (cpo)	A growth inhibitor of mycotoxin-producing fungi	*Pseudomonas pyrrocinia*	35S	About 60–70% reduction in *A. flavus* colony growth	Rajasekaran et al. (2000)
Tobacco	ZmPR-10	Rnase activity	*Zea mays*	d35S	Inhibited growth of *A. flavus*	Chen et al. (2006)
Zea mays	mod1	Ribosome-inhibiting protein (RIP) gene (called mod1)			Reduced *A. flavus* growth and aflatoxin contamination	Weissinger et al. (2003)

(continued)

Table 11.11 (continued)

Crop	Construct used for transformation				Performance	References
	Gene name	Gene function	Gene source	Promoter		
Zea mays	zhd101	ZEN-degrading enzyme	*Clonostachys rosea*	Act1 promoter	Reduced ZEN levels in the transgenic maize seed	Igawa et al. (2007)
Zea mays	α-amylase inhibitor protein	A growth inhibitor of mycotoxin-producing fungi	*Lablab purpureus*		Block the α-amylase activity of *A. flavus*,inhibit spore germination and fungal growth, and also reduce aflatoxin contamination	Chen et al. (2014)
Zea mays	Tachyplesin1-derived synthetic peptide AGM182	Synthetic antimicrobial peptide	Synthetic peptide	Ubiquitin-1 promoter	72% reduction in fungal growth as well as aflatoxin levels reduced by 76–98% reduction	Rajasekaran et al. (2018)
Arachis hypogaea	D5C	Antimicrobial peptide	*Zea mays*		Due to peptide toxicity, transgenic peanut callus showed poor recovery of plants	Weissinger et al. (1999)
Arachis hypogaea	Loxl	Lipoxygenases	*Glycine max*	Carrot embryo-specific promoter (DC3)	Showed reduced aflatoxin content	Ozias-Akins et al. (1999)

(continued)

Table 11.11 (continued)

Crop	Construct used for transformation				Performance	References
	Gene name	Gene function	Gene source	Promoter		
Arachis hypogaea	Tomato anionic peroxidase (tap1)				Confer aflatoxin resistance	Ozias-Akins et al. (2002)
Arachis hypogaea	Glucanase	Pathogenesis-related protein	*Nicotiana tabacum*	35S	Inhibited hyphal spread and aflatoxin contamination in the seeds	Sundaresha et al. (2010)
Arachis hypogaea	Rice chitinase Gene rcg3	Pathogenesis-related protein	*Oryza sativa*	35S	Transgenics showed 0–10% *A. flavus* infection	Prasad et al. (2013)
Arachis hypogaea	Lipoxygenase (PnLOX3)	Lipoxygenases	*Arachis hypogaea*			
Arachis hypogaea	Defensins	Antimicrobial peptide	*Medicago sativa*	FMV	Reported stable and high level of resistance to aflatoxin in transgenics up to 0–4 ppb	Sharma et al. (2017)
Arachis hypogaea	Forisomes		*Pisum sativum*			Bhatnagar et al., unpublished
Arachis hypogaea	Non-heme chloroperoxidase gene (cpo-p)	A growth inhibitor of mycotoxin-producing fungi	*Pseudomonas pyrrocinia*	d35S	Inhibit *A. flavus* hyphal spread and reduce aflatoxin accumulation	Niu et al. (2009)

(continued)

Table 11.11 (continued)

Crop	Construct used for transformation				Performance	References
	Gene name	Gene function	Gene source	Promoter		
Cotton	Non-heme chloroperoxidase gene (cpo-p)	A growth inhibitor of mycotoxin-producing fungi	*Pseudomonas pyrrocinia*	d35S	Inhibit *A. flavus* hyphal growth	Jacks et al. (2004), Rajasekaran et al. (2008a)
Cotton	Defensin D4E1 gene	Antimicrobial peptide	Synthetic peptide	d35S	Regulate the fungal growth and spread in cotyledons of both mature and immature cottonseed	Rajasekaran et al. (2005)
Cotton	Trypsin inhibitor protein	A growth inhibitor of mycotoxin-producing fungi	*Zea mays*	d35S	Expression of TIP in cottonseed is not high enough to prevent *A. flavus* colonization	Rajasekaran et al. (2008a)

It has been suggested that cuticular wax, tannin content, and chemical composition of the pericarps and embryos in peanut have key role in the inhibition of fungal invasion by *A. flavus* and aflatoxin formation (Liang et al. 2003). In vitro studies have shown that purified chitinase from peanut, Tex6 Maize kernel, and sugarbeet suppressed the fungal growth (Jwanny et al. 2001; Moore et al. 2004) which was attributed to increased chitinolytic activity and activation of other defense-related mechanisms. Overexpression of rice *chitinase* gene, *glucanase* gene, *Mod-1*, a synthetic version of maize ribosome-inactivating protein gene (a proteolytically activated form of RIP-1), bacterial *chloroperoxidase* gene, *PR10* family putative-resistant gene (ARAhPR10), and *defensins* have shown to confer enhanced resistance to *A. flavus* and aflatoxin contamination in peanut (Wilkinson et al. 2007; Niu et al. 2009; Sundaresha et al. 2010; Prasad et al. 2013; Xie et al. 2013; Sharma et al. 2017).

Several antifungal proteins have been identified through molecular breeding approaches, of which major proteins such as chitinases, β-1,3-glucanases, ribosome-inactivating proteins (RIPs), and *Zea*matin identified from maize kernels, rachis, and silk tissues play a crucial role in aflatoxin resistance (Guo et al. 1997; Chen et al. 1998; Lozovaya et al. 1998). A 14 kDa trypsin inhibitor protein from maize and 36 kDa protein AILp from hyacinth bean (*Lablab purpureus*) inhibit the α-amylase activity of *A. flavus*, thereby suppressing fungal growth and aflatoxin contamination in vitro (Chen et al. 1998; Fakhoury and Woloshuk 2001). Stress-related protein, glyoxalase I was reported to regulate magnesium levels inside maize kernels that stimulate *aflR* gene expression, thereby directly inhibiting aflatoxin accumulation (Chen et al. 2004).

Schubert et al. (2015) reported threefold increase in resistance to *A. flavus* infection compared with control lines through transgenic expression of the spined soldier bug (*Podisus maculiventris*) 21 amino acid thanatin AMP in maize. However, they have not evaluated the levels of aflatoxin content in transgenic lines. More recently, expressing the synthetic peptide *AGM182* conferred enhanced resistance to aflatoxin in maize showing 72% reduction in fungal growth and 76–98% reduction in aflatoxin levels in kernel screening assay using a highly aflatoxigenic *A. flavus* strain (AF70) (Rajasekaran et al. 2018). However, in this study, even the most superior transgenic maize line was reported to have aflatoxin levels of 60–150 ng/g under optimal conditions, which is beyond the permissible limits of 4–20 ng/g. Occasionally, while only one seed in thousand may be contaminated, it can produce a high aflatoxin value for the entire seed batch. Nonetheless, a very low to negligible aflatoxin content of 0–4 ppb was reported in peanut by using HIGS approach in peanut (Sharma et al. 2017).

Different antifungal proteins have been overexpressed in cotton plants using bacterial chloroperoxidases such as *CPO-P* (Jacks et al. 2004), a defensin *D4E1* gene (Rajasekaran et al. 2005), and maize kernel trypsin inhibitor protein (TIP) (Chen et al. 1998; Rajasekaran et al. 2008a, b). Transgenic cotton with the *TIP* gene under the control of enhanced double CaMV 35S constitutive promoter has shown enhanced resistance to *Verticillium*, but not *A. flavus* (Rajasekaran et al. 2008a, b), thereby suggesting a need for higher seed-specific expression of this gene in cotton seed. A non-heme chloroperoxidase gene (*cpo-p*) from *Pseudomonas pyrrocinia* that cat-

alyzes the conversion of alkyl acids to peracid by hydrogen peroxide (Jacks et al. 2000) was used to develop transgenic peanut and cotton plants where their progenies expressing *cpo-p* gene exhibited inhibition of *A. flavus* hyphal growth in vitro (Niu et al. 2009; Rajasekaran et al. 2008a, b).

A few studies were undertaken to alter the seed lipid pools for sporulation and aflatoxin formation using lipoxygenases in several crop species. Soybean *loxl* gene encodes an enzyme that catalyzes the formation of a specific lipoxygenase metabolite of linoleic acid, 13(S)-hydroperoxyoctadecadienoic acid (HpODE) that has been shown to suppress the aflatoxin biosynthetic pathway in vitro (Burow et al. 1997). Overexpression of soybean *loxl* gene in transgenic peanut under the control of an embryo-specific promoter from carrot enhanced aflatoxin resistance (Ozias-Akins et al. 1999). Expressing *PnLOX3*, a lipoxygenase gene from peanut, enhanced resistance to aflatoxin when overexpressed in peanut. Further greenhouse studies indicated no difference in pre-harvest *A. flavus* infection between transgenics and untransformed controls, while transgenics showed significant reduction in aflatoxin content compared to controls. This indicates that diverse mechanisms of resistances can control *A. flavus* infection and aflatoxin accumulation in peanut (Bhatnagar-Mathur et al. unpublished results). Additionally, pyramiding insect resistance gene with different antifungal gene constructs has been demonstrated to delay resistance to aflatoxigenic fungi in vitro, in situ, or *in planta*. Introduction of synthetic *cry1Ac* gene under the control of a CaMV 35S promoter into peanut cultivar MARC I dramatically reduce in vitro leaf feeding by lesser corn stalk borer (Singsit et al. 1997; Ozias-Akins et al. 2002). Further field screening of selected lines with *cry1Ac* also showed lower pod damage and reduced aflatoxin contamination (Ozias-akins et al. 2002). However, aflatoxin contamination was not directly correlated with damage due to the pink bollworm in cotton (Bock and Cotty 1999).

11.9.2 Host-Induced Gene Silencing-Mediated Resistance to Aflatoxin

Successful downregulation of fungal genes through RNAi depends on efficient uptake of siRNAs by the fungus as has been demonstrated on the movement of RNA between plants and fungi (Panstruga 2003). Interaction between fungal pathogens and their corresponding host occurs via haustoria which act as an interface for signal exchanges and nutrient uptake (Panstruga 2003; Voegele and Mendgen 2003; Micali et al. 2011). These close interactions assist in movement of mRNA signals between the host plant cells and fungal pathogens that leads to RNA silencing-mediated resistance in crop plants (Duan et al. 2012). Given the potential targeted downregulation of the aflatoxin pathway genes through host-induced gene silencing (HIGS), targeted degradation of specific fungal target sequences has been utilized to control fungal

growth and aflatoxin production (Nowara et al. 2010; Tinoco 2010; Yin et al. 2011; Koch et al. 2013). Gene silencing in *A. flavus* using inverted repeat transgenes (IRT) construct allows easier genetic manipulation of both intractable and tractable fungi as IRT need not be targeted to any specific location within the genome to function, and also there is no need to identify the flanking regions, promoter, or terminator of a gene. Furthermore, it is feasible to silence several genes with one construct. IRT construct containing sequences of aflatoxin pathway genes (*AflR*), suppressed aflatoxin production in *A. flavus* (Hammond and Keller 2005; McDonald et al. 2005) and (*AflD*) (Abdel-Hadi et al. 2011).

Silencing of gene expression through RNAi for functional gene validation and aflatoxin resistance has been reported in maize and peanut (Table 11.12). RNAi interference-based downregulation of *PR10* in maize increased sensitivity to heat stress, fungal growth, and aflatoxin production (Chen et al. 2010). This study showed reduction of *PR10* transcript levels by 65–99% and the corresponding PR10 protein levels by 61–81%. Similarly, knockdown of TI (trypsin inhibitor) in maize reduced the TI transcript levels and increased susceptibility to *A. flavus* colonization and aflatoxin production (Chen et al. 2016). Both these studies showed negative correlation of *PR10* and *TI* gene expression with aflatoxin production, respectively. Similarly, maize expressing hairpin RNA (hpRNA) cassette containing *stcA*, *stcJ,* and *aflR* sequences reported inhibition of aflatoxin biosynthesis (Alakonya and Monda 2013). Knockdown of transcriptional activator gene *aflR* maize showed significant reduction (14-fold vs. control plants) of aflatoxin content in maize kernels (Masanga et al. 2015). However, transgenic RNAi plants showed stunting and reduced kernel placement due to off-target effects of the siRNAs produced from the hpRNAs.

RNAi-based concurrent silencing of five aflatoxin biosynthetic, transport, or non-ribosomal peptide synthetase (NRPS) related genes (*aflR*, *aflS*, *aflC*, *aflep*, and *pes1*) led to 100% reduction in aflatoxin contamination in immature peanut kernels (Arias et al. 2015; Power et al. 2017). In another report, RNAi-based silencing of *aflC* gene (encoding for polyketide synthase, *pksA*) showed a significant reduction in the levels of aflatoxin ($\leq$93 ppb) in transgenic corn (Thakare et al. 2017). More recently, increased resistance to aflatoxin contamination in peanut has been reported by functional inhibition of the ver-1 (*aflM*) and *omtA* (*aflP*) genes through RNAi (Sharma et al. 2017). In yet another report, silencing of alpha-amylase gene *amy1* showed reduced fungal colonization and aflatoxin accumulation in maize inbred B104 expressing an RNAi construct targeting the *A. flavus* (Gilbert et al. 2018). Recently, mutant studies in *A. flavus* contributed to valuable information on the genes responsible for conidiation, sporulation, sclerotial production, virulence, and aflatoxin biosynthesis. Several reports on targeted disruption of fungal genes have accumulated over recent years to get insight into molecular mechanisms involved in pathogenesis. Some of these genes have been functionally validated (Table 11.13).

Table 11.12 List of genes encoding key enzymes/proteins used in RNAi-mediated aflatoxin resistance

Crop	Construct used for transformation				Performance	References
	Gene name	Gene function	Gene source	Promoter		
Zea mays	PR10	Pathogenesis-related protein	*Zea mays*	d35S	Significant increase in fungal colonization and aflatoxin production	Chen et al. (2010)
Zea mays	AflR	Aflatoxin regulatory gene	*A. flavus*	Ubi1	14-fold reduction in aflatoxin contents (vs. control) in the kernels derived from RNAi lines; stunting and reduced kernel placement phenotypes of transgenic plants	Masanga et al. (2015)
Arachis hypogaea	aflS, aflR, aflC, pes1, aflep	Aflatoxin regulatory and pathway, transport gene	*A. flavus*	35S	Showed 100% reduction in aflatoxin B1 and B2 in the seeds	Arias et al. (2015)
Zea mays	14 kDa trypsin inhibitor (TI)	A growth inhibitor of mycotoxin-producing fungi	*Zea mays*	d35S	Increased aflatoxin production in transgenics compared to negative controls	Chen et al. (2016)
Zea mays	aflC	Aflatoxin pathway gene	*A. flavus*	ϒ-zein endosperm-specific promoter	No toxin detected in RNAi transgenic maize kernel	Thakare et al. (2017)

(continued)

Table 11.12 (continued)

Crop	Construct used for transformation				Performance	References
	Gene name	Gene function	Gene source	Promoter		
Arachis hypogaea	aflM, aflP	Aflatoxin pathway gene	*A. flavus*	d35S	Decreased aflatoxin content in groundnut up to 1–4 ppb	Sharma et al. (2017)
Zea mays	Alpha-amylase gene amy1	Secreted proteins that catalyze hydrolysis of starch into sugar	*A. flavus*	d35S	Reduced fungal colonization and aflatoxin contamination in maize kernels	Gilbert et al. (2018)
Zea mays	*ZmPRms*	Pathogenesis-related protein	*A. flavus*	Zein promoter	Increased ~250–350% *A. flavus* infection and ~4.5–7.5-fold higher accumulation of aflatoxins in transgenics compare to control plants	Majumdar et al. (2017a, b)

Table 11.13 List of key genes as a candidate for RNAi-based gene silencing

Target gene	Encoded function	Effect of mutation on growth, sporulation or sclerotial formation	Effect of mutation on mycotoxin production	Effect on gene expression	References
Vea	Globar regulator, mediate AF activation by binding to laeA	Inhibited sclerotial formation	Loss of aflatoxin production	Suppress expression of several genes particularly aflatoxin biosynthetic pathway genes	Cary et al. (2007)
DmtA methyltransferase	A putative C-5 cytosine methyltransferase has role in DNA methylation	Reduced conidiation and altered sclerotia development	Reduced aflatoxin (AF) production	Suppress the transcriptional level of *aflC*, *aflK* and *aflO*, along with the regulatory genes, *aflR* and *aflS*, genes in the aflatoxin cluster	Yang et al. (2016a, b)
RmtA, a Putative Arginine Methyltransferase	rmtA is a global regulator that control expression veA and various cellular processes such as oxidative stress	Reduce conidiation but promotes sclerotial development	Reduced aflatoxin production	Reduced expression of aflM/ver1 and aflS/aflJ	Satterlee et al. (2016)
rtfA	*rtfA* controls the expression of the global regulators veA and laeA	Produce small conidiophores with reduced conidiation and sclerotial production	Reduced aflatoxin production	Increase expression of veA and laeA after 3 dai and then decrease after 4–5 dai	Lohmar et al. (2016)

(continued)

Table 11.13 (continued)

Target gene	Encoded function	Effect of mutation on growth, sporulation or sclerotial formation	Effect of mutation on mycotoxin production	Effect on gene expression	References
NsdC and NsdD	Global transcription factor that regulate asexual reproduction and secondary metabolism in *Aspergillus flavus*	Perturbed conidiophore development, premature conidiation, and a lack of sclerotial development	Reduced aflatoxin production	Reduced expression of aflD, aflM and aflP, and aflR is expressed at normal level	Cary et al. (2012), Gilbert et al. (2016)
Caleosin-Like Protein	Calcium-binding proteins endowed with peroxygenase activities, as well as co-oxidation reactions and reduction of polyunsaturated fatty acid hydroperoxides	Reduced conidiation	Reduced aflatoxin production	Reduce expression of aflR and aflD	Hanano et al. (2017)
Adenylate Cyclase AcyA	Core component of cAMP signaling	Cause severe defects in fungal growth, sporulation, and sclerotia formation	Reduced aflatoxin production	Reduced expression of lae, aflR, and aflO	Yang et al. (2016a, b)
Amy1, the a-amylase gene	Catalyze hydrolysis of starch into sugar	Reduced fungal growth	Reduced aflatoxin production	Not reported	Fakhoury and Woloshuk (1999)
High-affinity phosphodiesterase PdeH	Controls the specificity, amplitude, and temporal duration of cAMP signaling	Reduced conidiation and sclerotia formation	Increased aflatoxin production	Increased expression of aflD and aflR	Yang et al. (2017)

(continued)

Table 11.13 (continued)

Target gene	Encoded function	Effect of mutation on growth, sporulation or sclerotial formation	Effect of mutation on mycotoxin production	Effect on gene expression	References
Fungi-specific velvet family protein VelC	Regulates development and secondary metabolism in filamentous fungi	Increased conidiation and inhibited production of cleistothecia		Increased expression of brlA, abaA, wetA and vosA involved in sporulation	Park et al. (2014)
Homeobox gene, hbx1	Well conserved transcription factors, that control development and differentiation	Complete loss of conidiation and sclerotia formation	Reduced production of aflatoxins B1 and B2, aflatrem and cyclopiazonic acid	Reduce expression of regulatory genes genes*brlA* (bristle) and *abaA* (abacus), for conidiophore development together with aflC, aflD, aflM and the cluster-specific regulatory gene, aflR	Cary et al. (2017)
aflK	Encode versicolorin B synthase that converts versiconal to versicolorin B		Showed significantly reduced sclerotial production and biosynthesis of aflatoxin in *A. flavus*		Cary et al. (2000a, b, c)
Spermidine synthase (*spds*) gene	Polyamine (PA) metabolic gene spd controls hypusination-mediated activation of eukaryotic translation initiation factor 5A (eIF5A)	Complete loss of fungal growth and sporulation	Reduced aflatoxin production	Increased expression of PA metabolic genes such as *spds, samdc*, *odc*, and *spms*	Majumdar et al. (2018)

(continued)

Table 11.13 (continued)

Target gene	Encoded function	Effect of mutation on growth, sporulation or sclerotial formation	Effect of mutation on mycotoxin production	Effect on gene expression	References
pecA gene	pecA gene encodes P2c polygalacturonase, having pectinase activity that degrades fungal cell wall	Caused significant reduction in fungal aggressiveness	Reduced aflatoxin production	Not reported	Shieh et al. (1997)
msnA	Oxidative stress regulatory gene	Retarded fungal growth with increased conidiation	Slightly increased aflatoxin production	Increased expression of superoxide dismutase, catalase, and cytochrome c peroxidase	Chang and Ehrlich (2010)

Many of the genes mentioned in the above studies can be potential targets to be explored for aflatoxin resistance in future using HIGS. Successful implementation of HIGS to control aflatoxin resistance has been reported by Arias et al. (2015) and Sharma et al. (2017); whereas, Masanga et al. (2015) reported negative effect of RNAi silencing construct on growth and yield of all maize transformants. This indicates that it may be the result of off-target effect of siRNA produced from the hpRNAs. Hence, efficacy and off-target effect of siRNA have been the major concerns for developing a successful HIGS against fungal pathogens. However, with the advancement in the area of functional genomics, availability of genome sequence data and new bioinformatics tools enable design and engineering of effective dsRNA expression constructs addressing concerns of off-target silencing. Several web-based computational tools such as siRNA Scan (http://bioinfo2.noble.org/RNAiScan.htm), siDirect 2.0 (http://siDirect2.RNAi.jp/) (Naito et al. 2009; Naito and Ui-Tei 2012) are now publicly available to design appropriate RNAi construct to prevent off-target silencing. This software was effectively used to identify potential off targets during PTGS in plants (Xu et al. 2006). By carefully designing the sequences to be used for HIGS and targeting conserved gene in *A. flavus*, off-target silencing of unintended genes in the host plants as well as in the beneficial plant-associated organisms, such as mycorrhizas, rhizobia and biocontrol species, such as *Trichoderma* species can be avoided.

11.10 Conclusions and Future Prospects

Population of this planet is rapidly growing that is projected to cross 9 billion in the next 20 years, making it challenging to produce enough food for such a huge population. To meet the ever-increasing food requirement, development of new and novel crop improvement technologies would be highly advantageous. Aflatoxin contamination occurs worldwide mainly in tropical and subtropical regions such as Africa, Asia, and Latin America due to favorable conditions such as drought stress and high temperature in these regions. The contamination of food commodities by aflatoxin is a unique problem in agriculture and is not likely to be completely resolved by conventional control strategies routinely used against other fungal pests of plants. Not only being a food safety issue in many lower-income or developing countries, aflatoxin contamination of key staple crops causes significant post-harvest losses leading to trade losses (Lewis et al. 2005; Mutegi et al. 2013).

Since aflatoxin resistance is a multigenic trait, substantial control of aflatoxin contamination necessitates a multidisciplinary approach for action. It includes variety of cultural practices, host resistance, molecular breeding approaches, transgenic approaches, and RNAi silencing that minimize aflatoxin production at both pre- and post-harvest stages. Further, chemical (ammoniation and nixtamalization), physical

(removal of damaged or incomplete kernels/seed), and biological strategies contribute to the degradation of aflatoxins from contaminated foods and feeds. Current control practices to eliminate fastidious fungal parasites of plants such as those involved in race-specific interactions are considerably less effective in controlling facultative pathogens such as *A. flavus* and *A. parasiticus.* The most effective and practical approaches include improved cultural practices as well increased host resistance. Post-harvest practices in proper handling and storing grain are precarious tools to prevent the development of mycotoxins.

The development and use of host resistance offer a possible and cost-effective solution. However, limited genetic diversity in the germplasm makes conventional breeding approaches challenging as high levels of natural resistance to aflatoxin accumulation have not been identified in peanut, cotton, and maize. While in maize, innate resistance to aflatoxigenic fungi is present, and it has not been suitably exploited because of its typical polygenic nature and the poor agronomic performance of resistance sources. Although modern approaches of molecular genetics are supporting researchers and breeders to exploit native resistance, currently available commercial hybrids usually lack sufficient resistance levels. The identification of resistance sources in the cultivated and wild germplasm needs to be improved using robust screening techniques that produce reproducible results. Moreover, better understanding of *A. flavus* and aflatoxin genetics, identification of resistance genes and use of cost-effective, accurate and reproducible aflatoxin bioassays facilitate improvement of host resistance to aflatoxin contamination (Mahuku et al. 2013).

Although aflatoxin biosynthesis has been extensively studied by various researchers, the pathway is complex and many steps are still not well understood. Current 'omic' approaches such as proteomics and transcriptomics are being exploited to find the proteins/genes that contribute to the aflatoxin resistance. Although large number of genes have been identified in plants in response to *A. flavus* infection, unraveling the relationship between overexpressed genes and aflatoxin resistance is crucial for successful development of aflatoxin tolerant crops. Extensive research is underway to understand the role of various regulatory networks that associates aflatoxin biosynthesis to the perturbations of cell metabolism, and in particular to oxidative stress. Moreover, missing enzymatic steps in aflatoxin biosynthesis must be discovered in order to put the aflatoxin biosynthetic puzzle together.

More recently, RNA-seq (RNA-sequencing) has been used for genomewide gene expression analysis and to determine gene structures and expression profiles in maize in response to *A. flavus* providing greater visions and higher precision than microarrays. With recent development in sequencing technologies and bioinformatics strategies, in 2016, the genome sequences of *A. duranensis* and *A. ipaensis* were officially released, which would provide new opportunities to link the aflatoxin-resistant phenotype with genes. This would facilitate marker-assisted selection (MAS) for quick indirect selection of the target gene by using molecular markers closely linked to a target gene and quantitative trait loci (QTLs) analysis to improve aflatoxin resistance

in elite cultivars. QTLs that expressed stably across different genotypes and under multiple environments will be immensely useful in breeding for aflatoxin resistance. Further, markers identified from these QTL regions that are conserved and additive in nature have the potential to facilitate transfer of genes from resistant to susceptible line.

While many biocontrol agents have been tested for their efficacy to reduce aflatoxin contamination of crops, only atoxigenic *A. flavus* strains have been used commercially. Biological control using atoxigenic *A. flavus* strains to prevent further infection by toxin producing strains has proved very efficient, consistently reducing aflatoxin contamination by >80% (Bandyopadhyay et al. 2016). In view of the potential genetic recombination in *A. flavus*, precautions are needed to have stable biocontrol strains and protect against the unintended introduction of *A. flavus* strains that could inflict an extra burden on food safety and quality. Understanding the mechanisms of gene regulation in aflatoxin biosynthesis is crucial for identifying the natural inhibitors of *Aspergillus* growth and aflatoxin production. Significant improvement in cloning of aflatoxin pathway genes in the fungal genetic engineering will help in production of stable aflatoxin non-producers and for optimization of strain competitiveness for future use in biocontrol applications. Nonetheless, the major shortcoming to the wide dissemination of the biological control strategy has been the need to develop and register a product for each country, as strains cannot be used across borders; thus, making the process costly and cumbersome (Bandyopadhyay et al. 2016).

Therefore, a more targeted approach to eliminate key events in the *A. flavus* infection and/or aflatoxin biosynthetic process might be more efficient in reducing or eliminating aflatoxin contamination. Noteworthy developments made in our understanding of mechanism of aflatoxin biosynthesis and regulation have allowed development of transgenic plants with silencing vectors for the expression of self-complementary hairpin RNAs of antifungal/aflatoxin reducing genes. A number of reports have successfully demonstrated the use of HIGS (RNAi) as a means of down-regulating expression of genes implicated in the regulation of aflatoxin biosynthesis and pathway that lower aflatoxin contamination in food crops (Arias et al. 2015; Bhatnagar-Mathur et al. 2015; Masanga et al. 2015; Thakare et al. 2017; Sharma et al. 2017; Gilbert et al. 2018). Nonetheless, successful RNAi-mediated control would depend on many factors such as RNAi specificity, dsRNA regulation and movement of dsRNA and sRNAs from host to pathogen and vice versa, prediction of off-target effects, and stability of sRNA in fungi (Majumdar et al. 2017a, b). Moreover, effectiveness of HIGS under post-harvest storage and field conditions must be determined under low moisture stress which could possibly make seeds dormant, and therefore might affect the stability of hpRNAs/siRNAs (Majumdar et al. 2017a, b; Gressel and Polturak 2018).

Another alternative non-transgenic RNAi approach is spray-induced gene silencing (SIGS) which exploits the RNAi mechanism through the exogenous application of long dsRNA and siRNAs. It has been reported to be effective in controlling both *B. cinerea* and *F. graminearum* (Koch et al. 2016; Wang et al. 2016a, b). Compared to HIGS, although this procedure does not require development of stably transformed

plants, the effect of SIGs application is not long lasting, and hence exploring applications of double layered hydroxide clay nanosheets loaded with dsRNA has potential for its persistence over longer periods (Mitter et al. 2017). Since the prevalence of mycotoxins within the food web is an unavoidable and critical situation the world is going through, apart from good hygenic measures cognizant efforts must be made to denote the aflatoxin poisoning in human and livestock. With the aid of biotechnological tools, researchers all over the globe have geared up to bring on plate a much safer food that is free of aflatoxins.

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Chapter 12
Reducing the Acrylamide-Forming Potential of Crop Plants

Sarah Raffan and Nigel G. Halford

Abstract Acrylamide is a food-processing contaminant formed from free asparagine and reducing sugars during high-temperature cooking and processing. It is a Group 2A carcinogen, and EFSA's CONTAM Panel has expressed concern for the potential tumour-inducing effects of dietary exposure. Fried, baked, roasted and toasted potato, coffee and cereal products are the major contributors to dietary acrylamide intake. The European Commission has recently introduced strengthened risk management regulations for acrylamide in food, including compulsory mitigation measures and new Benchmark Levels. Steps taken by manufacturers to reduce acrylamide formation in potato chips in Europe resulted in a 53% decrease from 2002 to 2011. However, since 2011 there has been a levelling off, suggesting that the easy gains have already been made and further large reductions are unlikely. The acrylamide-forming potential of potatoes is influenced by seasonal and geographical factors, making regulatory compliance for potato products more difficult. In cereals, acrylamide formation is determined by free asparagine concentration: this differs substantially between varieties but is also very responsive to environmental factors and crop management. Ensuring good disease control and sulphur sufficiency are particularly important. The relationship between precursor concentration and acrylamide formation is more complex in potato, with the concentration of reducing sugars the most important parameter in most datasets but free asparagine concentration contributing to the variance. Storage is a key issue for potatoes due to the phenomena of cold and senescent sweetening. Investigations into the genetic control of acrylamide formation in wheat have focussed on asparagine metabolism, in particular asparagine synthetase, while biotech potatoes with reduced expression of asparagine synthetase and vacuolar invertase are already on the market in the USA.

Keywords Wheat · Rye · Potato · Acrylamide · Processing contaminant · Food safety · Asparagine · Reducing sugars

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12.1 Introduction

Acrylamide (C_3H_5NO) is an odourless, organic, water-soluble compound that was discovered in a range of cooked foods in 2002 as a food-processing contaminant (Tareke et al. 2002). It has been classified as a probable (Group 2a) human carcinogen (International Agency for Research on Cancer 1994), based on rodent toxicological data. It also shows developmental and neurotoxic effects (Friedman 2003; CONTAM Panel 2015). It can be ingested, inhaled or absorbed through the skin and is one of the carcinogens found in cigarette smoke.

Acrylamide forms a non-toxic polymer, polyacrylamide, which has a wide range of industrial uses, from sewage treatment and water purification to the production of food packaging and cement. More familiarly to molecular biologists and biochemists, it is used to make polyacrylamide gels for the separation of protein or nucleic acid molecules by polyacrylamide gel electrophoresis (PAGE). Polyacrylamide is usually contaminated with small amounts of the monomer, and because of polyacrylamide's use in water treatment, monomeric acrylamide is a potential water pollutant. The World Health Organisation has, therefore, set a tolerance level of 0.5 μg per l [equivalent to 0.5 parts per billion (ppb)] for the presence of acrylamide in drinking water, with the understanding that levels should be as low as reasonably achievable. The level of 0.5 μg per l is arguably below the limit of detection, and essentially the discovery of any acrylamide in water should trigger a pollution alert and an investigation.

Acrylamide can be metabolized via epoxidation to produce glycidamide ($C_3H_5NO_2$), and it has been suggested that glycidamide is actually responsible for the carcinogenic and genotoxic effects attributed to acrylamide. Both acrylamide and glycidamide form adducts with glutathione, and these are further converted to mercapturic acids, which are excreted from the body. This is the main detoxification route preventing either chemical from accumulating in the body.

12.2 Toxicology

The toxicological effects of acrylamide have been studied in detail in a range of animal species, including monkeys, cats, dogs, guinea pigs, rats and mice, with the most comprehensive evidence coming from rodent studies. The European Food Safety Authority (EFSA)'s Panel on Contaminants in the Food Chain (CONTAM) analysed the results of these studies, summarizing the findings in a report published in 2015 [European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM) 2015]. The Panel concluded that acrylamide showed neoplastic and genotoxic effects and at high doses could cause developmental and neurological changes.

Evidence for the toxicity of acrylamide in humans came first from studies on the effects of occupational exposure, with a 1993 study on workers in an acrylamide-

manufacturing factory in China showing damage to their peripheral nervous system (Bergmark et al. 1993). A later study (Bergmark 1997) showed that laboratory workers who regularly performed PAGE showed higher levels of acrylamide in their systems than controls. Importantly, these early studies revealed unexplained high levels of acrylamide in control groups, indicating that there must be another source of acrylamide exposure. The explanation for this came in 2000, when it was shown that rats that were fed fried animal feed for 1–2 months showed a higher level of acrylamide in their system than rats fed on unfried feed (Tareke et al. 2000). The implication was that acrylamide was forming when the feed was fried. Acrylamide exposure is measured by the formation of haemoglobin–acrylamide adducts in the blood, and the amount of acrylamide in the feed correlated with the levels of these adducts. Perhaps surprisingly in retrospect, this study passed under the radar and it was a subsequent study in 2002 that alerted the world to the presence of acrylamide in food. That study reported the discovery of acrylamide in a wide range of common cooked foods (Tareke et al. 2002), with the highest levels in fried potato products and crispbreads. Subsequently, cereal products and coffee were also shown to be major contributors to dietary intake.

12.3 The Levels of Acrylamide in Food

Acrylamide is not present at all in raw food and can, therefore, be classified as a processing contaminant, defined as a substance that is produced during cooking or processing, is not present or is present at much lower levels in raw, unprocessed foods, and is undesirable because it is potentially harmful to consumers or has adverse effects on product quality (Curtis et al. 2014b). Acrylamide does not form during boiling, but is associated with fried, baked, roasted and toasted foods. The main contributors to dietary acrylamide intake in Europe are potato, coffee and cereal products (CONTAM Panel 2015), with potato chips (called crisps in the UK), French fries, biscuits, breakfast cereals, bread (particularly if it is toasted), crispbreads and all types of coffee affected. These foods contain acrylamide in the tens or hundreds of μg per kg (ppb). Based on data collected by the European Food Safety Authority (EFSA 2011), cereal and potato-based products each contribute approximately 40% of dietary intake in Europe, while the other 20% is attributed to coffee. The largest single contributor is bread, which contains relatively low levels of acrylamide unless it is toasted but is eaten in large quantities.

12.4 Formation

Following the discovery of acrylamide in popular foods, acrylamide was shown to form from free (soluble, non-protein) asparagine and reducing sugars within the Maillard reaction (Mottram et al. 2002; Stadler et al. 2002). Reducing sugars and

free asparagine can, therefore, be regarded as the precursors for acrylamide, although the carbon skeleton of acrylamide is derived entirely from asparagine. The major reducing sugars in potato tubers are the monosaccharides, glucose and fructose. Cereal grains also contain the disaccharide maltose, but very little maltose is present in potatoes. Sucrose, while not itself a reducing sugar, should also be considered with respect to acrylamide-forming potential because it is so abundant in plant tissues and can be hydrolysed to form glucose and fructose.

The Maillard reaction was first described by Louis Camille Maillard in 1912 (Maillard 1912), although the steps in the reaction as they are understood today were proposed half a century later by an American chemist, John Hodge (1953). The reaction as Hodge described it comprises a series of non-enzymatic reactions between sugars and amino groups, principally those of free amino acids, and any amino acid can participate, not just asparagine. It is promoted by high temperature and low moisture content, and its products include melanoidin pigments, which are complex polymers responsible for the brown color in fried, baked, roasted and toasted foods, and a plethora of compounds that impart flavor and aroma. The compounds that are formed depend on the amino acid and sugar composition of the food and the cooking/processing conditions, and they give different cooked foods their characteristic color, flavor and aroma.

Acrylamide forms in the reaction as a result of a Strecker-type degradation of asparagine. The asparagine reacts with a carbonyl compound intermediate to produce a Schiff base (a compound containing a carbon-nitrogen double bond in which the nitrogen atom is attached to an organic group). This is then converted to acrylamide by decarboxylation followed by either the removal of a substituted imine or the elimination of a carbonyl group to produce an intermediate, 3-aminopropionamide, which is then converted to acrylamide by the removal of ammonia. This model for the conversion of asparagine to acrylamide was proposed by Zyzak et al. (2003), and the role of 3-aminopropionamide as a potent intermediate in acrylamide formation has been confirmed (Granvogl and Schieberle 2006; Granvogl et al. 2007).

It is important to state that the reaction is multi-step, with free amino acids participating in the early and final stages. This means that there may be complex relationships between precursor (free asparagine and reducing sugar) concentration and acrylamide formation, and other free amino acids can drive the early stages of the reaction but compete with asparagine in the final stages. It is also important to note that because color, flavor and aroma compounds form by similar chemical pathways to acrylamide, any action taken to reduce acrylamide formation may also affect product quality.

While the Maillard reaction appears to be the major route for acrylamide formation in food, others have been proposed. For example, acrylamide has been shown to form in wheat gluten even after the removal of starch and all the soluble components, which would include the simple sugars and free amino acids (Claus et al. 2006b). It also forms in dried fruit, such as prunes and dates, and while elevated temperatures are used in drying systems to produce prunes, dates and other dried fruits, these rarely exceed 60 °C, so the source of the acrylamide in these products remains unknown.

12.5 Regulations

The discovery of acrylamide in food in concentrations many times the tolerance level set for drinking water presented regulatory authorities with an extremely difficult problem. In 2004, the Joint FAO/WHO Food Standards Programme CODEX Committee on Food Additives and Contaminants issued a discussion paper on acrylamide requesting the FAO/WHO Joint Expert Committee on Food Additives (JECFA) to comment on the extent to which acrylamide was bioavailable in food and its safety implications. JECFA issued its opinion in 2006 (JECFA 2006), stating that while adverse neurological effects were unlikely at the estimated average dietary exposure, morphological changes in nerves could not be excluded for individuals with high exposure. In addition, for a compound that was both genotoxic and carcinogenic, the margins of exposure indicated a health concern. It recommended that 'appropriate efforts to reduce concentrations of acrylamide in food should continue'. JECFA issued a second opinion in 2011, making almost identical statements and recommendations.

In 2005, EFSA's CONTAM Panel endorsed JECFA's risk assessment on acrylamide in food and recommended that appropriate efforts to reduce acrylamide concentrations in foodstuffs should continue. The Panel requested that data on acrylamide levels in food should be collected over at least a three-year time span and reported once a year to EFSA who would compile a database so that the effectiveness of measures adopted to reduce the formation of acrylamide in food could be assessed. In May 2007, therefore, the European Commission issued Recommendation 2007/331/EC on the monitoring of acrylamide levels in food (European Commission 2007), requiring that European Union Member States should perform annual monitoring of acrylamide levels in foodstuffs in 2007, 2008 and 2009 and provide the data to EFSA.

EFSA reported its analysis of the 2007 data in 2009 (EFSA 2009) along with acrylamide results for 2003–2006 that had been collected from Member States and compiled by the Commission's Joint Research Centre's Institute for Reference Materials and Measurements. EFSA concluded that it was not clear if the mitigation measures that had been adopted were effective.

The European Commission responded again in 2010 with Commission Recommendation 2010/307/EU on the monitoring of acrylamide levels in food (European Commission 2010). This required Member States to continue to collect data on acrylamide levels in food on a regular basis. Also in 2010, EFSA released its analysis of the 2008 data (EFSA 2010), with the conclusion that any trend towards lower levels of acrylamide was limited to certain food groups. Once again, the European Commission responded, this time with Recommendation C (2010) 9681 Final of January 2011 on investigations into the levels of acrylamide in food (European Commission 2011). This required the competent authorities in Member States to investigate why the acrylamide levels in some foodstuffs should be 'significantly higher than the levels in comparable products of the same product category'. The Recommendation included Indicative Values (Table 12.1) for the presence of acrylamide in food, with

Table 12.1 Indicative Values and Benchmark Levels for acrylamide in food, set by the European Commission (2011, 2013, 2017)

Food	Benchmark Level 2017 (ppb)	Indicative Value 2013 (ppb)	Indicative Value 2011 (ppb)
French fries	500	600	600
Potato chips (UK crisps)	750	1000	1000
Soft bread (wheat)	50	80	150
Soft bread (other)	100	150	
Breakfast cereals: bran products, whole grain cereals, gun puffed grain	300	400	400
Breakfast cereals: wheat and rye based	300	300	
Breakfast cereals: maize, oat, spelt, barley and rice based	150	200	
Biscuits	350	500	500
Crackers	400	500	500
Crispbread	350	450	500
Gingerbread	800	1000	–
Cereal-based baby foods	40	50	100
Baby foods (not cereal based) without prunes	–	50	80
Baby foods (not cereal based) with prunes	–	80	
Biscuits and rusks for infants and young children	150	–	250
Roast coffee	400	450	450
Instant coffee	850	900	900
Coffee substitute (cereal-based)	500	2000	–
Coffee substitute (chicory)	4000	4000	–

varying Indicative Values applied to different food types, based on the monitoring data compiled by EFSA. If a product was found to exceed the Indicative Value for its food type, an investigation by the relevant food safety authority was triggered to find out why. The results of the investigation were reported to the commission, and action was taken to ensure that the manufacturer addressed the problem.

In May 2011, EFSA reported the full results of acrylamide monitoring from 2007 to 2009, and provided estimates of the exposure levels of Europeans to dietary acrylamide (EFSA 2011). Mean acrylamide levels in the 2009 data ranged from 37 ppb for soft bread to 1504 ppb for substitute coffee, while the highest single level was found in a potato chip sample at 4804 ppb. Trends towards lower acrylamide levels were apparent in crackers, infant biscuits and gingerbread, but not in any of the other food categories. The mean exposure of Europeans to dietary acrylamide was estimated to be 0.31–1.1 μg per kg bodyweight per day for adults, 0.43–1.4 μg per kg bodyweight per day for adolescents (11–17 years old), 0.70–2.05 μg per kg bodyweight per day for children (3–10 years) and 1.2–2.4 μg per kg bodyweight per day for toddlers (1–3 years).

Another EFSA report followed in 2012 (EFSA 2012), once again finding little change in acrylamide levels over the period from 2007 to 2010, with Indicative Values exceeded in between 3 to 30% of samples in different food categories. The conclusion that the measures adopted by industry to reduce acrylamide levels in foods were not working prompted calls for the European Commission to take stronger action, and revised and in many cases reduced Indicative Values (Table 12.1) were introduced through Commission Recommendation 2013/647/EU of November 2013 (European Commission 2013). This Recommendation required Member States to continue to work with food businesses to investigate products that exceeded the new Indicative Value for that product type. It also made reference to an 'updated risk assessment' being performed by EFSA on the presence of acrylamide in food. EFSA produced its risk assessment in the document 'Scientific Opinion on acrylamide in food', prepared by the CONTAM Panel (2015), which we have referred to several times already. The report is long and comprehensive, and the conclusions of the Panel on the risk posed by dietary acrylamide were that 'the margins of exposure indicate a concern for neoplastic effects based on animal evidence'. This assessment effectively forced the hand of the European Commission, which embarked on another process of strengthening its risk management regulations for acrylamide. New proposals were issued in June 2017, approved by the European Commission, Council and Parliament, then published in November 2017 as Commission Regulation (EU) 2017/2158 (European Commission 2017). The Regulation came into force on 11 April 2018.

The Regulation paraphrases the 2015 CONTAM Panel report with starker language, stating that the Panel's assessment had 'confirmed previous evaluations that acrylamide in food potentially increases the risk of developing cancer for consumers in all age groups'. It replaces Indicative Values with Benchmark Levels, which are lower than the corresponding Indicative Value for almost all product types (Table 12.1), justifying the reduction by describing Benchmark Levels as performance indicators rather than triggers for investigation. The Regulation also states that Benchmark Levels will be regularly reviewed by the Commission with the aim

of setting lower levels and holds out the threat of imposing Maximum Levels (i.e. levels of acrylamide above which it would be illegal to sell a food product) in the future. It includes annexes in which mitigation measures that have been developed to reduce acrylamide formation are described, from variety selection through crop management to a variety of measures that have been shown to be effective during food processing. These are effectively codes of practice, and the wording of the Regulation makes it clear that the adoption of these measures is compulsory: food business 'shall' rather than 'should' apply the mitigation measures that are set out. However, at the time of writing, it is not clear what sanctions may be applied to a food business when one of its products is found to be above the relevant Benchmark Level and the business cannot show that the appropriate mitigation measures were applied.

In our view, the direction of travel for the European Commission on acrylamide remains inexorably towards tighter regulation, with the very real possibility that it will eventually lead to the imposition of Maximum Levels. More detail on the Commission's thinking regarding Maximum Levels for acrylamide was provided when the issue was discussed at a meeting of the European Parliament's Environment, Public Health and Food Safety Committee in January 2017. It was made clear that the intention was to impose Maximum Levels on sectors of the food industry that do not show 'sufficient progress' in reducing acrylamide in their products. Although this is frustratingly vague (what is meant by 'sufficient progress', for example?), we advise anyone in the food production and supply chain to take the threat to impose Maximum Levels seriously.

Another regulator to have taken action is Jerry Brown, who was the Attorney General of the State of California in 2005, and filed a lawsuit against potato chip and French fry manufacturers (Heinz HJ, Frito-Lay, Lance Inc and Kettle Foods) along with Procter and Gamble and four fast-food chains (McDonald's, Burger King, KFC and Wendy's) for selling potato chips and fries without a Proposition 65 warning to alert consumers to the presence of acrylamide. Proposition 65 is California's 'Safe Drinking Water and Toxic Enforcement Act' and requires businesses to post warnings of any chemical in their products that may cause cancer. The lawsuit was settled in 2008 when the manufacturers committed to cut the level of acrylamide in their products and payed $3 million in fines. The fast-food chains also agreed to display acrylamide warnings at their restaurants.

Another case in California is still ongoing. In 2010, the Council for Education and Research on Toxics (CERT), a small not-for-profit organization, brought a lawsuit under Proposition 65 against Starbucks and 90 other companies, demanding that the coffee industry remove acrylamide from its products or alert consumers to the presence of acrylamide through warning signs and/or labels. Removing acrylamide altogether from coffee is currently not possible, but in March 2018, a judge of the California Superior Court, County of Los Angeles, issued a preliminary decision in the case in favour of CERT.

There has been less action at the Federal level in the USA. The Food and Drug Administration (FDA) has to date not set any restrictions on levels of acrylamide in food, although it has issued an 'action plan' with the goals of developing screening

methods, assessing dietary exposure and identifying means to reduce it, as well as increasing understanding of acrylamide toxicology (Food and Drug Administration 2016).

In Canada, acrylamide has been added to the list of chemicals in the government's Chemicals Management Plan, and Health Canada has stated that it is committed to collaborating with the food industry to further pursue reduction efforts for acrylamide in processed foods. It has also implemented an acrylamide monitoring program to evaluate the effectiveness of the reduction strategies it recommends and to assess industry compliance, with the possibility of setting 'reduction targets' in the future. Food authorities in Japan and Hong Kong, and Food Standards Australia New Zealand (FSANZ), the binational agency with responsibility for food standards in Australia and New Zealand, have taken similar stances. The position of these authorities, therefore, could be described as somewhat behind the European Commission but heading in the same direction.

12.6 The Acrylamide Toolbox

Acrylamide reduction became one of the most important targets for the food industry after 2002 and has remained so ever since. In Europe, food companies have shared their knowledge as they have developed methods to reduce acrylamide formation, and the information has been compiled in an Acrylamide Toolbox published by FoodDrinkEurope [formerly the Confédération des Industries Agro-Alimentaires de l'UE (CIAA)]. The first Acrylamide Toolbox was published in 2005, and the most recent update was published in late 2019 (FoodDrinkEurope 2019).

The Acrylamide Toolbox is available online to download for free. The methods it describes encompass agronomy, the use of additional ingredients in the recipe, reduction in pH by addition of citric or ascorbic acid, dilution of precursor concentration by incorporation of raw materials with lower concentrations of free asparagine and/or reducing sugars, pre-treatment with asparaginase to reduce asparagine concentrations, improved control of thermal input and moisture during processing, pre-treatment by washing and blanching to remove sugars, and quality control by the elimination of dark-colored products by inline optical sorting (color being a good indicator of acrylamide levels, since both melanoidin pigments and acrylamide form in the Maillard reaction).

These measures have been applied with varying degrees of success to different product types, with none proving effective in all types of products, and some affecting product quality as well as reducing acrylamide formation. Nevertheless, and somewhat ironically for the food industry, many of the measures from the Acrylamide Toolbox now appear in the annexes of European Commission Regulation (EU) 2017/2158 and are effectively compulsory. For potato chips, at least, it is possible to demonstrate that the measures have been successful because European potato chip manufacturers affiliated to the European Snacks Association have made data on acrylamide levels in their products available for analysis. Two studies have been

published, one covering the period from 2002 to 2011 (Powers et al. 2013) and the other a longer period from 2002 to 2016 (Powers et al. 2017). These showed a 53% reduction in mean acrylamide levels from 763 ppb in 2002 to 358 ppb in 2011. However, the trend did not continue past 2011, with the mean acrylamide level flattening out and the level in 2016 of 412 ppb being slightly higher than that of 2011. This indicates that the easiest and most effective methods to reduce acrylamide levels had already been implemented by 2011 and further improvements may be difficult to achieve without reductions in the acrylamide-forming potential of the raw material.

The studies also highlighted two factors that make it more difficult for food producers to achieve regulatory compliance on a consistent basis. The first of these is a seasonal effect, arising from the fact that European potatoes are harvested between July and October, and for the rest of the year are used from storage. Potatoes in storage are prone to increases in reducing sugar concentration, caused by cold and senescent sweetening (see Sect. 12.7.3). The second factor is a geographical one, with potatoes grown in northern Europe (Denmark, Finland, Lithuania, Latvia, Norway and Sweden) for some reason having a higher acrylamide-forming potential than potatoes grown elsewhere. The two factors combine to raise the mean acrylamide levels in chips produced in that region so that the proportion of samples with more than 750 ppb acrylamide (the Benchmark Level set for chips by the European Commission) is over 30% for the whole of the first half of the year, peaking at more than 45% in May (Fig. 12.1). Even for the other regions of Europe, the 'failure rate' of chips with respect to the 750 ppb Benchmark Level in some months of the year is over 10%. Clearly, if a Maximum Level of 750 ppb were imposed on chips, it is not being over-dramatic to say that the industry would not be able to continue as things stand.

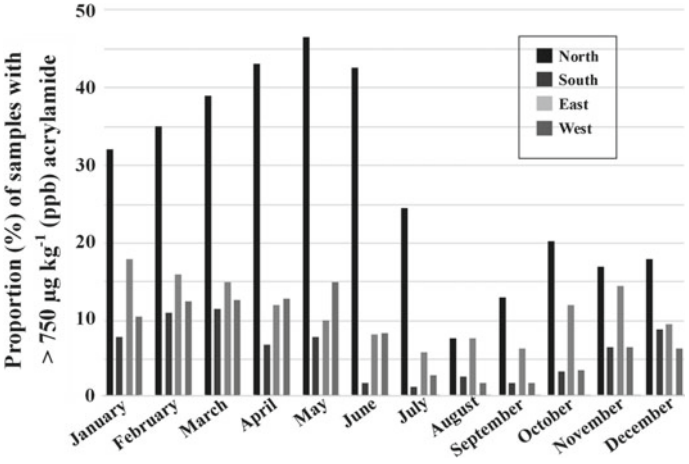


Fig. 12.1 Proportion (%) of samples of potato chips produced in Europe with more than 750 μg kg^{-1} (ppb) acrylamide for each month over the period 2011–2016 for geographic regions, North, South, East and West. Plotted from data provided by Powers et al. (2017)

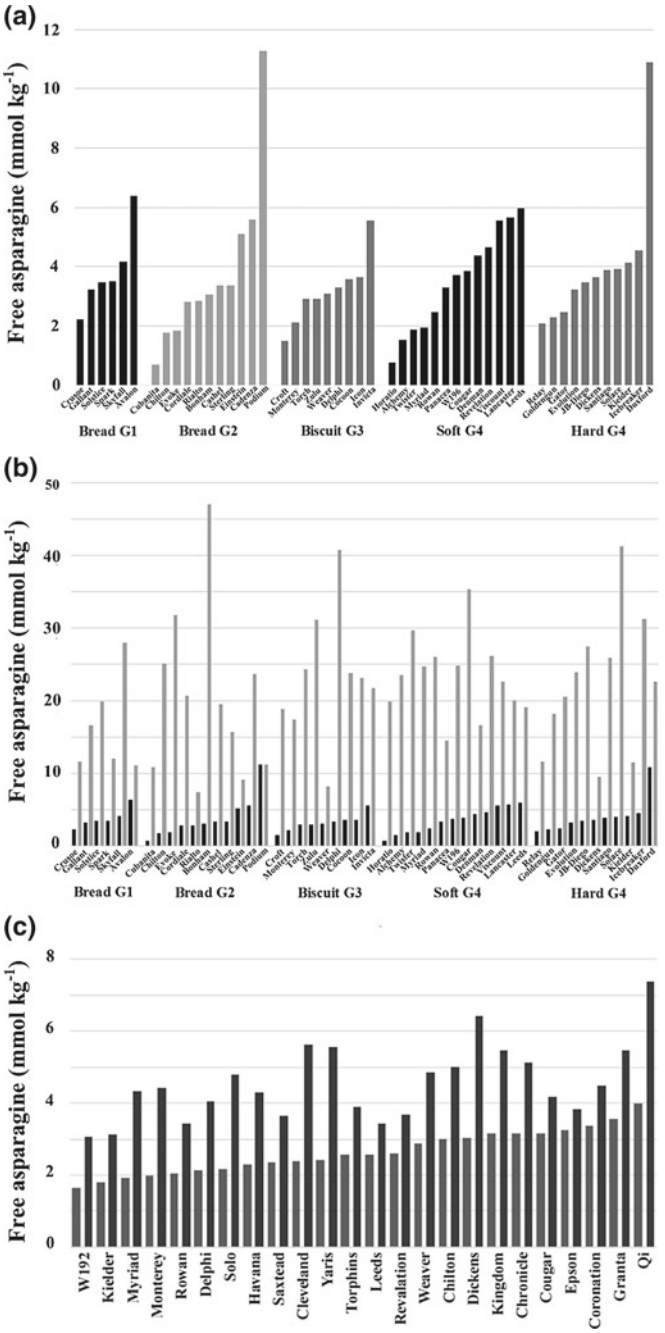
12.7 Genetic and Agronomic Approaches to Reducing the Acrylamide-Forming Potential of Wheat, Rye and Potato

Research aimed at developing strategies to tackle the problem of acrylamide formation in food products from the crop raw material side has been directed at the genetic control (G) of precursor concentration in crop plants, the environmental factors (E), including crop management, that influence the accumulation of precursors, and the interactions between them ($G \times E$). Approaches taken range from the identification of current crop varieties with low acrylamide-forming potential through the development of best agronomic practice and the application of modern genomics and biotechnology.

12.7.1 Variety Selection

In cereal grain, the determinant of acrylamide-forming potential is free asparagine concentration (Muttucumaru et al. 2006; Granvogl et al. 2007; Curtis et al. 2009, 2010, 2016, 2018b; Postles et al. 2013) and a consideration of the free asparagine content in cereal-derived raw materials is included in the acrylamide mitigation measures for cereal products in European Commission Regulation (EU) 2017/2158 (European Commission 2017).

Free asparagine accumulates to high concentrations in many plant species during normal physiological processes, such as seed germination, and in response to a range of abiotic and biotic stresses, including salt, drought and nutritional stress (Lea et al. 2007). Indeed, it can become the predominant free amino acid in cereal grains under some stress conditions, and this is an example of how stress can have profound effects on crop composition (Halford et al. 2015), with implications in this case for food safety. The responsiveness of free asparagine concentrations to extraneous factors means that varietal rankings with respect to free asparagine concentration in the grain may change from one site to another or from one harvest year to another. Nevertheless, the range in free asparagine concentration across different varieties growing at the same site can be many fold, so variety selection can make a huge difference to acrylamide-forming potential. Figure 12.2a for example, shows the free asparagine concentrations in the grain of 50 varieties of wheat grown in a field trial in the UK in 2012–2013 (Curtis et al. 2018b). Furthermore, it has been possible to identify a number of consistently low varieties over multiple field trials in the UK. These are Claire, Cocoon, Cordiale, Croft, Delphi, Horatio, Monterey and Myriad. Of these eight varieties, Delphi, Claire, Cocoon, Croft and Monterey are all soft biscuit types (classified as Group 3 in the UK), while Horatio and Myriad are soft Group 4 types (used predominantly for animal feed and bioethanol production, but suitable for some grists). The eighth variety, Cordiale, is classified as having bread-making potential (Group 2). It is important to note that some soft wheat varieties have


(a)
Free asparagine (mmol kg-1)
12
10
8
6
4
2
0
Bread G1
Bread G2
Biscuit G3
Soft G4
Hard G4
(b)
Free asparagine (mmol kg-1)
50
40
30
20
10
0
Bread G1
Bread G2
Biscuit G3
Soft G4
Hard G4
(c)
Free asparagine (mmol kg-1)
8
6
4
2
0
W192
Kielder
Myriad
Monterey
Rowan
Delphi
Solo
Havana
Saxtead
Cleveland
Yaris
Torphins
Leeds
Revalation
Weaver
Chilton
Dickens
Kingdom
Chroniele
Cougar
Epson
Coronation
Granta
Qi

◀**Fig. 12.2** Graphs illustrating measures that can be taken to control the acrylamide-forming potential of wheat. **a** Mean free asparagine concentration in the grain of 50 varieties of winter wheat grown in a field trial in the UK, in 2012–2013 (Curtis et al. 2018b). The varieties are grouped according to milling type. **b** Mean free asparagine concentration in the grain of the same 50 varieties grown in split-plots in which half the plot was supplied with sulphur (black columns), while the other half was not (grey columns) (Curtis et al. 2016). **c** Mean free asparagine concentration in 24 varieties of winter wheat grown in a field trial in the UK in 2011–2012 and either treated with fungicides (grey) or left untreated (black) (Curtis et al. 2016)

relatively high concentrations of free asparagine in the grain, and selecting grain for processing simply on the basis of it being from a soft-milling variety, expecting it to have low acrylamide-forming potential, is not advisable.

Despite the identification of varieties with consistently low free asparagine concentration in the grain, food processors face a number of problems in using variety selection to help address the acrylamide problem. The annual introduction of multiple new varieties to the market in the UK and the dropping of others, for example, means that by the time there is enough data on the free asparagine concentration of a variety over different harvest years and sites the variety may no longer be available. The only solution to this problem is for free asparagine concentration to be measured during variety development, but at present this does not happen. Another problem for food businesses is that they operate across national borders, and information on the levels of free asparagine in the grain of different wheat varieties is unavailable for most countries.

Despite these problems, we strongly advise wheat breeders and farmers to engage on the acrylamide issue and take it into consideration when selecting genotypes to take forward in breeding programmes or choosing varieties for cultivation. The cheapest and most straightforward measure that processors could take to reduce the acrylamide levels in their products, or make the levels more consistent and predictable, would be to switch variety, so breeders and farmers must be ready for that.

Variety selection is already being used to reduce the amount of acrylamide in rye products. The rye supply and processing chain in the UK is much smaller than that of wheat, and rye is more likely to be grown to contract. Possibly for that reason, there are anecdotal reports that processors are already stipulating that specific, low asparagine varieties should be grown.

The potato supply chain is in a similar position, with farmers generally growing to contract. Potato varieties have been bred for different specific end uses, and the wide range in acrylamide-forming potential of different varieties and types (chipping, French fry or boiling) (Fig. 12.3) (Halford et al. 2012; Muttucumaru et al. 2013, 2014; Elmore et al. 2015) led to Regulation (EU) 2017/2158 stipulating that food businesses must use potato varieties that are suitable for the product type, where the content of fructose, glucose and free asparagine is the lowest for the regional conditions. In potato, the relationship between precursor concentration and acrylamide formation is more complex than in cereals, with the concentration of reducing sugars the predominant factor in acrylamide-forming potential but free asparagine contribut-

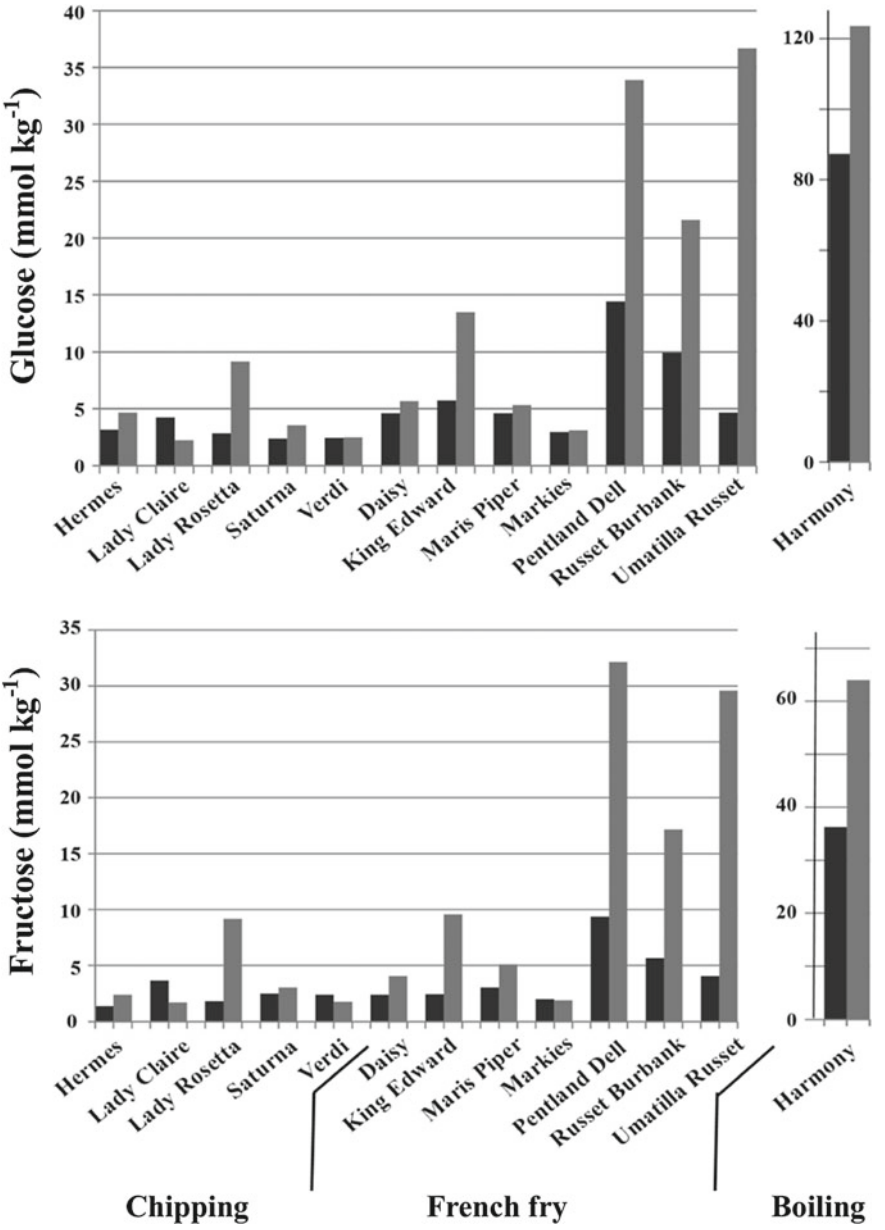


Fig. 12.3 Concentrations of glucose and fructose in 13 potato varieties grown in a field trial in the UK in 2010 and sampled before a 6-month storage period (dark grey) and after storage (light grey) (Muttucumaru et al. 2014). The varieties are separated into chipping, French fry and boiling type

ing to the variance in some datasets (Muttucumaru et al. 2014). The influence of free asparagine increases as the ratio of free asparagine to reducing sugars decreases (Muttucumaru et al. 2017), which means that free asparagine concentration is more important in French fry than chipping varieties.

12.7.2 Crop Management

Two crop management measures have been identified as being crucial for managing the acrylamide risk of wheat, and have been written into Commission Regulation (EU) 2017/2158. These are ensuring that the wheat crop receives an appropriate supply of nitrogen and sulphur, and that it is adequately protected from disease. Sulphur deficiency, in particular, causes a massive accumulation of free asparagine in wheat grain, with a concomitant effect on acrylamide formation in heated flour (Fig. 12.2b) (Muttucumaru et al. 2006; Granvogl et al. 2007; Curtis et al. 2009, 2018b), while nitrogen has the opposite effect (Claus et al. 2006a; Martinek et al. 2009). Nitrogen fertilizer is, of course, indispensable for farmers to obtain the maximum possible yield of cereal crops and the protein content and quality that are required for some end uses, notably bread production. Commission Regulation (EU) 2017/2158 simply states that the 'correct' amount should be applied. We also advise that sulphur should accompany nitrogen application to ensure that the nitrogen is incorporated into protein, which is the desired outcome, rather than being stored in the grain in the form of free asparagine, which is what happens when sulphur is not available. This has been standard practice in Sweden since the 1990s. We recommend a rate of sulphur application to wheat of 20 kg per hectare (equivalent to 50 kg SO_3 per hectare) (Curtis et al. 2014a), and this rate should be used for all wheat likely to enter the human food chain, not just wheat destined for bread-making.

Free asparagine also accumulates to high levels in many plant tissues in response to pathogen infection, and this has been shown to be the case for wheat grain (Martinek et al. 2009; Curtis et al. 2016) (Fig. 12.2c). Consequently, Commission regulation 2017/2158 states that food businesses 'shall ensure application of good practices on crop protection measures to prevent fungal infection'.

No similar study on the effect of disease on acrylamide-forming potential has been performed with rye or potato. However, experiments have been performed on the effect of nutrition. In rye, nitrogen has a similar effect to that seen in wheat, with substantial increases in free asparagine accumulation with more nitrogen application (Postles et al. 2013, 2016). However, sulphur deficiency does not induce the dramatic response observed in wheat. This may be because rye is better at scavenging sulphur from the soil, but rye also appears to respond differently, reducing the amount of nitrogen that it allocates to the grain when sulphur is not available rather than accumulating the nitrogen in the grain in the form of free asparagine (Postles et al. 2013).

The effect of nitrogen fertilization on the concentration of reducing sugars in potato tubers had been investigated before the acrylamide issue arose because of the

implications of reducing sugar concentration for fry color. It was reported in 1990, for example, that potatoes grown under high nitrogen had lower amounts of free sugars, and consequently tended to have less fry color (Roe et al. 1990). The discovery of acrylamide in food reignited interest in the topic, but different studies have produced inconsistent results, with one, for example, confirming the earlier findings (de Wilde et al. 2006) and another reporting no effect of nitrogen application or other agricultural practice (Amrein et al. 2003). Our own investigations on 13 varieties of potato grown in a field trial in 2010, and treated with different combinations of nitrogen and sulphur, revealed a more complicated picture than the previous studies, with nitrogen having type- and variety-specific effects on glucose concentrations and sulphur having a direct effect, bringing about a reduction of 26% from zero to 40 kg per hectare application. There was a trend for substantial increases in free asparagine accumulation in all the varieties in response to nitrogen fertilization, but sulphur had no consistent effect on any of the free amino acids. In other words, the dramatic effect of sulphur deficiency on free asparagine concentrations in wheat does not occur in potato. The complex, variety-specific changes observed in potatoes in response to the treatments led us to conclude that advice on both nitrogen and sulphur application would have to be carefully tailored for each variety.

A study of the effect of irrigation and drought stress on potatoes had a similarly somewhat frustrating outcome (Muttucumaru et al. 2015). The conclusion from the study was that different potato genotypes were affected in dissimilar fashion by the availability of water, indicating that there is no single, unifying potato tuber drought stress response. Farmers were advised to irrigate potatoes only if necessary to maintain the health and yield of the crop, because irrigation could increase the acrylamide-forming potential of the tubers.

12.7.3 Potato Storage

While it has proved difficult to develop clear advice for farmers on the crop management measures that they can take to reduce the acrylamide-forming potential of potatoes, we can be very clear on how potatoes should be handled post-harvest. Potatoes are biochemically quite active during storage and are prone to both cold and senescent sweeting, in which glucose and fructose accumulate. Cold sweetening occurs at storage temperatures around and just above 4 °C, while senescent sweetening occurs as the potatoes begin to break dormancy and prepare to sprout, defining the end of the optimum storage window for a particular variety. Both cold and senescent sweetening are associated with vacuolar invertase (VInv) activity, while senescent sweetening is also driven by the breakdown of starch through the actions of phosphorylase L (PhL) and starch-associated R1 (R1). A storage temperature of 8.5–9.5 °C is typical of commercial potato stores, representing a compromise between colder temperatures that would promote cold sweetening and warmer temperatures that would encourage the potatoes to sprout. Even at these temperatures, the concentrations of reducing sugars and, therefore, acrylamide-forming potential of many varieties

increases through storage (Fig. 12.3) and varieties that show good stability with respect to sugar concentrations are being favoured for use after long-term storage.

Commission Regulation 2017/2158 stipulates a storage temperature above 6 °C and that potatoes must be stored in the appropriate conditions for each variety and used within their optimum storage window. The humidity of the store must be controlled to minimize senescent sweetening, while sprouting must be suppressed 'using appropriate agents'. 'Appropriate agents' might include chemical sprout suppressants, such as chlorpropham (isopropyl 3-chlorocarbanilate; $C_{10}H_{12}ClNO_2$). However, these too are coming under scrutiny and at the time of writing it looks likely that products containing chlorpropham will soon cease to be authorized for use in the European Union. It is not clear how stored potatoes will be managed without those products.

The temperature of 6 °C stipulated in the regulation is arguably too low to prevent cold sweetening. Even so, while it will not affect most commercial potato stores, it may come as a shock to retailers because up to now they have been used to keeping potatoes at 4 °C in a cold store with other fresh produce. This practice will have to stop. It is also important that consumers are made aware that the domestic refrigerator is not an appropriate place to store potatoes.

12.7.4 Genetic Interventions to Reduce Acrylamide-Forming Potential

If breeders are to be able to develop cereal varieties with low acrylamide-forming potential they will need information on the genetic control of asparagine synthesis and accumulation so that appropriate targets for genetic intervention can be identified. To this end, a network has been compiled representing the molecular factors involved in asparagine metabolism, consisting of 212 nodes (genes, enzymes or molecules) and 246 edges (reactions between nodes) (Curtis et al. 2018a). The core enzymes involved are nitrate reductase, nitrite reductase, glutamine synthetase, asparagine synthetase, asparaginase, aspartate kinase, aspartate amino transferase, glutamate dehydrogenase, ferredoxin-dependent glutamate synthase, NADH-dependent glutamate synthase and glutamate decarboxylase. Any of these may be suitable targets for genetic interventions, but the obvious starting point is asparagine synthetase.

Asparagine synthetase catalyses the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine. There are four asparagine synthetase genes in wheat: *TaASN1*, *TaASN2*, *TaASN3* and *TaASN4* (Gao et al. 2016). *TaASN4* was only discovered from wheat genome data and has still not been cloned or characterized, although recently we have obtained evidence that it is not highly expressed in the grain (Curtis et al. 2019). Of the other genes, the expression of *TaASN2* in the embryo and endosperm of the grain during mid to late grain development has been shown to be by far the highest of any of the genes in any tissue (Gao et al. 2016).

Four asparagine synthetase genes have also been identified in maize (Todd et al. 2008), while initially five genes were described in barley (Avila-Ospina et al. 2015). A detailed analysis of the wheat gene family using genome data for the variety Chinese Spring confirmed the presence of four asparagine synthetase genes, but while *TaASN1*, *TaASN2* and *TaASN4* were all single copy genes, located on Chromosomes 5, 3 and 4, respectively, there were two copies of *TaASN3* (*TaASN3.1* and *TaASN3.2*) on Chromosome 1. Reanalysis of the barley genes suggests that the fifth gene identified in that species was, in fact, a second copy of *HvASN3*, suggesting that four asparagine synthetase genes may be typical of the cereals. This analysis also showed that wheat variety Chinese Spring lacked a *TaASN2* gene on the B genome. This may be important when it comes to breeding for wheat with less asparagine synthetase activity in the grain. Some varieties do have this gene, but Chinese Spring is not unique because varieties Claire, Paragon and Robigus also lack it.

In order to confirm that *TaASN2* is responsible for most of the asparagine synthetase activity in wheat grain, *TaASN2* and the next most highly expressed asparagine synthetase gene in the grain, *TaASN1*, were expressed in *Escherichia coli*, so that the biochemical properties of the enzymes they encode could be compared (Xu et al. 2018). Both genes expressed active asparagine synthetases, able to synthesize asparagine and glutamate from aspartate and glutamine. Both also continued to produce glutamate even when aspartate had run out, indicating that the removal of the amino group from glutamine to produce glutamate could proceed independently of the transfer of that amino group to aspartate to produce asparagine. This was consistent with a model proposed by Gaufichon et al. (2010), in which the reaction stages occur sequentially rather than concurrently. Modelling of the reactions catalysed by the TaASN1 and TaASN2 enzymes showed them to be biochemically very similar. This, coupled with the gene expression data, led to the conclusion that TaASN2 was the major enzyme synthesizing asparagine in wheat grain and an appropriate target for genetic interventions.

Breeders could use this information in traditional ways, by investigating the amount of variation in the *TaASN2* gene between different varieties and associating it with high or low free asparagine concentration in the grain. Certainly, the absence of a *TaASN2* gene on Chromosome 3B could be exploited. However, the hexaploid nature of wheat means that, even though there is a single *TaASN2* gene in each haploid genome, there are four to six in total across all three genomes, depending on the presence or absence of the gene on Chromosome 3B, and the effects of allelic variation in any one gene may be masked and easy to miss.

A more direct approach would be to use biotech techniques to target all of the *TaASN2* genes in one go, using RNA interference or a genome editing technique such as CRISPR-Cas9 or TALENS. The genes could also be knocked out using chemical or radiation mutagenesis and screening of mutant populations for mutations in the *TaASN2* genes. These mutations would then have to be stacked to produce a *null* line. Success with any of these techniques would require free asparagine concentration to be reduced enough to have a significant effect on the acrylamide-forming potential of the grain while grain yield, nitrogen content and protein quality were unaffected.

Reduced asparagine synthetase gene expression has already been achieved in potato. Indeed, varieties Innate® and Innate® Generation 2, produced by the Simplot Company of Boise, Idaho, are both already on the market in the USA. Potato has two asparagine synthetase genes, *StASN1* and *StASN2*, and the expression of both was initially reduced in a popular American French fry variety, Ranger Russett, using RNAi (Rommens et al. 2008). This resulted in potatoes that accumulated as little as 5% of the free asparagine present in the unmodified controls, but while the plants grew normally under glass, they produced small, cracked tubers when grown in the field. This problem was overcome by targeting *StASN1* alone and reducing its expression specifically in the tubers (Chawla et al. 2012). Plants carrying this trait produce normal yields of potatoes with very low concentrations of free asparagine, and it is this trait that has been incorporated into the Innate® and Innate® Generation 2 varieties.

Innate® and Innate® Generation 2 also have reduced expression of genes *PhL*, encoding phosphorylase L, and *R1*, encoding starch-associated R1, as well as reduced bruising through the targeting of gene *PPO5*, which encodes a polyphenol oxidase. Innate® Generation 2 also has reduced expression of a vacuolar invertase gene (*VInv*), as well as increased resistance to late blight through incorporation of a resistance gene, *Rpi-vnt1.1*, from a wild potato species, *Solanum venturii*. The low concentrations of free asparagine and reducing sugars in the tubers of Innate® Generation 2 are claimed to reduce acrylamide-forming potential by 90% compared with conventional potatoes. Such varieties with multiple novel traits and improved food safety show just how far ahead of Europe the USA is in crop biotechnology.

The vacuolar invertase (*VInv*) gene has also been targeted using TALENs in French fry variety, Ranger Russet (Clasen et al. 2016). Tubers from lines with a full knockout had undetectable levels of reducing sugars, and chips produced from them had much lower levels of acrylamide than chips produced from control tubers. Varieties carrying this trait have not been commercialized yet. It is possible that a genome editing technique was used in this case because there is no prospect at all of GM varieties being developed for the European market. However, at the time of writing, prospects for the commercialization of genome edited crop varieties in Europe also look bleak (Halford 2019).

12.8 Conclusions

The issue of acrylamide forming during the cooking and processing of popular foods has become one of the most difficult challenges facing the food industry. It is important that everyone in the food supply chain engages on the problem and takes action to ensure that they are not caught out as regulations are introduced and tightened. The food industry would benefit hugely from the breeding of new crop varieties with reduced acrylamide-forming potential. Biotech is likely to play a big part in this wherever biotech crops can be grown, and we have already seen the development and commercialization of biotech potatoes with hugely reduced acrylamide-

forming potential in the USA (Sect. 12.7.4). Progress, however, will depend on the full engagement of plant breeders, and we encourage plant breeders to adopt low acrylamide-forming potential as a breeding target if they have not done so already.

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Chapter 13
Phytate-Free Food Crops: Phytases—The Game Changers for Alleviation of Micronutrient Malnutrition and Improved Nutrition

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Abstract More than half of the world populations are affected by micronutrient malnutrition and one-third of world's population suffers from anemia and zinc deficiency, particularly in developing countries. Iron and zinc deficiencies are the major health problems worldwide. Populations that depend on legumes and grains as staple food consume diets rich in phytic acid. Inositol hexaphosphate, otherwise known as phytate, represents the major storage form of phosphorus and also contributes to the storage of trace elements in plants. Such a storage reservoir is also attributed to antinutritional activities in monogastric animals and plants by its strong chelation of Fe, Ca, and Zn to form insoluble complexes that cannot be absorbed via diet. This phenomenon contributes to Zn and Fe deficiency. The enzymatic degradation of phytate in the digestive tract or reduction of phytate levels in the food product or grains, when subjected to various processing strategies, has resulted in a varied level of phosphorylation. This uncertainty in rendering comprehensive nutrition enhancement in the population of the developing countries has made researcher lean on gene-editing strategies for alleviation of phytate content. This chapter gives an overview of phytic acid as an unbalanced nutrition provider, evaluation of phytate content, and the use of phytases to enhance micronutritional levels in plants.

Keywords Nutrition · Food · Developing countries · Iron · Zinc · Phytase

13.1 Introduction

Globally, agronomy plays a key role in provision of sufficient and nutrition-rich diet. Due to ever-increasing world population and shrinking cultivated lands, it is important to improve the quantity of food production. Plants are crucial source of

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calories for humans that provide prerequisite nutrition, yet only a handful of species are used for human consumption. Fruits and vegetables harvested directly from plants pose important sources of minerals, vitamins, fiber, and energy. As plant proteins are the primary dietary protein source for all humans and animals and are much less expensive to produce than animal protein, they will continue to play a dominant role in world food security. The nutritional quality of plant proteins can be improved by balancing the essential amino acids. This is because quality proteins not only benefit health and living, compared with low-quality proteins but also reduce the amount of consumption because of their rich and well-balanced essential amino acids supply. Understanding how plants harness, produce, and store health-beneficial nutrients is an innate goal of plant scientists. Rice, wheat, corn, beans, cassava, potato, sweet potato, sugar beets, and others are the dietary base for the world's population and represent important food staples for low-income communities. Besides being energy-dense and carbohydrate-rich, these crops are also an important entry point into the human diet for micronutrients, vitamins, and trace elements (both essential and toxic). Thus, breeding efforts to enhance crop nutrition, and avoiding accumulation of antinutrients, heavy metals, and toxic elements has direct impact on human health.

The purpose of agriculture is to provide sufficient food for all the people. With the ever-increasing world population and shrinking cultivated lands, it is important to improve the quantity of food production.

Genetic engineering and conventional breeding strategies are some of the plant modification strategies used for generating highly nutritious crop variety. Such fortification strategies usually target iron, zinc, and vitamin-A fueled by the report released by WHO stating a lack of these in the diet of the global human population. As there are only a few biofortified crops that have seen the light of day after the integration of basic research into final crop products (for e.g., seeds), it is still necessary to pursue this for global benefit. A report by De Moura and coworkers in 2016 revealed increasing the intake of vitamin-A by increasing the concentration of β-carotene. This report was made more effective by the undertaking of the crop variety with an effective program at the population level; reflecting the need-based research for uplifting the nutritional equivalence in inadequate populations. The requirement of a steady consumption of the biofortified product was confirmed by a clinical study that confirmed an increase in the retinol and β-carotene concentration on consumption of yellow cassava. The 'how-what-why' of the nutritional scope of plant products has been undertaken on a tremendous advanced level to understand the role of minerals and vitamins in plants so that they can be tapped for human benefit. An equivalent or a befitting role has also been played by plant breeders for improving crop plants for more than 1000 years to make varieties easy to grow, harvest, nutritious, and productive. Recent years have seen the integration of plant scientists and breeding techniques to help those suffering from nutrient deficiencies in a sustainable manner.

13.2 Manipulating Seeds to Remove Antinutritional Factors

The use of proteins in food industry as a part of vegetable proteins has been attributed to relate the behavior of foaming, emulsification, and gelation. Some studies have been carried out to alter the functional behavior of proteins for engineering the properties of legume globulins gave valuable insights to the functional and structural aspects of soybean proteins (Wang et al. 1998). Any lack of amino acids in seed globulins has been overcome by mixing them with cereal protein as a target for improvement (Shewry and Tatham 1997). Another attribute of proteins is that they are considered poor contributors to ruminant nutrition as the degradation of proteins occurs in the rumen. Therefore, additional processing measures such as heat treatments are necessary to improve the nitrogen values for these animals.

Improvement of protein levels in seeds is mainly associated with the removal of factors that generate potential allergens, undesirable flavours, and/or improve digestibility. Further, targets that result in improved functional behavior of the proteins are also undertaken. Directed improvement on these lines uses the integration of efforts to sufficiently define the molecular basis of improvement, genetic variation studies, and breeding by genetic manipulation. Alteration of the amino acid composition has resulted in the expression of sulfur-rich proteins by natural variation and genetic manipulation. Such approaches that tackle the deficiency problems by using natural approaches have resulted in the enhanced quality and yield of the seed protein. Improved understanding of the regulatory mechanisms involved in the seed proteins has been associated with the environmental effects and the genetic background of proteins in model legumes. Initial experiments confirmed the presence of many antinutritional properties associated with the albumin fraction which was then considered to be more favorable than the globulins. Although a few mutants targeting the albumin fraction have been identified, the bioavailability of these proteins in the seed or germinated plant is unclear. Mutants targeting the lipoxygenases in *Pisum sativum* and *Glycine max* (Forster et al. 1999) have not reported the loss of seed or seedling vigur shows a very interesting platform that can be banked on for further studies. The major pea seed albumin, PA2, has a number of characteristics that are undesirable for various end uses such as its cytosolic location and hydrophobic nature during germination and storage conditions. The insolubility of this protein is contributed by its free sulfhydryl group that resists the physiological mechanism of digestion in the tract of chicken (Crévieu et al. 1997). A protein homologous to PA2 has shown lectin-like properties in chickpea and has been deduced for allergic responses in individuals sensitive to chickpea. Removal of such proteins that contribute to allergy or other antinutritive effects is target for improving seed quality for feed and food. Variants without the antinutritive properties have also been introduced and checked for desirability for elite genetic backgrounds by molecular markers.

13.3 Phytic Acid as an Unbalanced Nutrition Provider

One of the most fascinating bioactive food compounds that are widely distributed in plants is phytic acid (Table 13.1). The molecular structure shows that phytic acid interferes with intestinal absorption by binding to polyvalent cations and inhibits the bioavailability of trace elements and minerals. Such molecules result in serious deficiencies in micronutrient levels and reflect negatively on the balance of nutrition especially in underdeveloped and developing countries. Phytic acid also contributes to antioxidative, anticancer activities which are of great importance in industrialized countries. But the contribution of the same compound to antinutrient does not favor the use of this molecule for the global population. In developing countries, where deficiencies of zinc and iron are widespread, prevention of deficiencies brought about such antinutritive molecules is to be pursued with utmost significance. A better-balanced supply of essential nutrients can be supplemented either by degrading food phytases or improving daily diet.

Myo-inositol 1,2,3,4,5,6-hexakisdihydrogen phosphate, also called phytic acid, is the major storage form of phosphorus. It comprises to about 5% by weight in legumes, cereals, seeds, and nuts which represents ~80% of total phosphorus in plants (Reddy et al. 1982; Vats and Banerjee 2004). The rapid accumulation of phytate is observed

Table 13.1 Content of phytate or phytic acid in various foods

Taxonomic names	Content of phytic acid/Phytate (in grams/100 g)
Zizania sp.	2.20
Triticale secale	>1.89
Pennisetum sp.	>1.67
Secale cereal	>1.46
Avena sativa	>1.16
Sorghum sp.	>3.35
Oryza sp.	>1.08 (in grain) and >3.91 (in bran)
Bran of *Triticum* sp.	>7.3
Germ of *Triticum* sp.	>1.35
Phaseolus vulgaris	>2.38
Vigna unguiculata	>2.90
Pisum sativum	>1.22
Lens culinaris	>1.51
Pinus pinea	~0.20
Macadamia integrifolia	>2.62
Juglans regia	>6.69
Pistacia vera	>2.83
Helianthus annuus	>4.3
Glycine max	>2.22

during the ripening period in leguminous seeds, oilseeds and grains of rice and wheat and corn. The location of phytate is found in the endosperm of corn, aleurone, and pericarp of the bran fraction in wheat. Electron-dense globoids are predominantly the storage organelles of phytic acid. Their presence in the aleurone layer of maize, wheat, and barley increases the size of seed when increased concentration of phytic acid is present in the grain. The absence of phytate-degrading enzymes in the digestive tract of monogastric animals makes the metabolism of phytic acid difficult (Boling et al. 2000). Therefore, inorganic phosphate is largely supplemented with inorganic phosphate to provide sufficient phosphorus requirement.

The mineral status of the foods consumed in the developing and underdeveloped countries has also been stimulated by the consumer's interest in the whole grain products and whole grains. The zinc, iron, and calcium deficiencies resulting due to the limited bioavailability of minerals are due to high phytate content. A balanced meal that includes food compounds such as organic acids, ascorbic acid, and food fermentation products competes with phytic acids in binding to trace elements and minerals, However, the food deficiency of these competitors in the diet of the underdeveloped countries does not inhibit the effects of phytic acid although little evidence exists from national or global nutritional surveys that factor the effect of such antinutritive molecules.

The chelating properties of phytic acid not only result in the binding of cations in seeds. Phytic acid also binds to minerals and makes them unavailable as nutritional factors when released during processing of feed or food in the gut of the animal or human. A couple of studies by Gharib et al. (2006) and Glahn et al. (2002) show inhibition of iron and zinc uptake inhibition. Various other studies also report the reduction in the intestinal absorption of zinc, calcium, iron, magnesium, and manganese but not copper. By utilizing the platform of CRISPR, biofortification of tomatoes has been undertaken by reducing the amount of phytic acid and increasing the antioxidant content of tomato fruits [Kaul et al. 2019; under review; Patent File No: 201711038417 (TEMP/E-1/39247/2017-DEL)]. Some processing treatments such as heat treatment, cooking/boiling, autoclaving, pressure cooking, microwave treatment, extrusion cooking, toasting, and soaking have been established with very minor effects to the content of phytic acid in grains.

13.4 Determination of Phytate Content

Phytate is subsequently estimated either by determining the phosphate, inositol, or iron content of the precipitate (direct method) or by measuring the excess iron in the supernatant (indirect method). These approaches are not specific for phytate due to the coprecipitation of partially phosphorylated myo-inositol phosphates and should, therefore, be limited to the analysis of material which contains negligible amounts of phytate dephosphorylation products. If substantial amounts of partially phosphorylated myo-inositol phosphates are present such as in processed foods, the content of phytate will be overestimated by using phytate determination methods based on

iron precipitation. More recently, high-performance liquid chromatography (HPLC) techniques have been introduced into phytate determination (Xu et al. 1992). Among these, ion-pair reverse-phase and anion-exchange chromatography are largely used today. These systems allow the simultaneous separation and quantification of myo-inositol tris- to hexakisphosphates (ion-pair reverse-phase chromatography) or myo-inositol mono- to hexakisphosphates (anion-exchange chromatography). Furthermore, a number of isomer specific ion-exchange chromatography methods with gradient elution for the separation and quantification of myo-inositol phosphates in the picomolar range have been developed very recently (Chen and Li 2003).

13.5 Phytases for Removal of Phytic Acid

Phytases are myo-inositol hexakisphosphate phosphohydrolases that achieve the hydrolysis of phytate to orthophosphate and lower the levels of substituted inositol phosphates. The use of these enzymes is beneficial to remove or reduce the maximum amount of phytic acid in seeds without affecting the mineral content of the grains. The activity of this enzyme is achieved by targeting the phosphomonoester bonds and liberating inorganic phosphates.

The ubiquitous presence of phytases in animals, plants, and microorganisms was established by many researchers over the years (Vats and Banerjee 2004; Rao et al. 2009; Yao et al. 2012). Distinct variation in the biophysical and biochemical properties has been associated with the source or expression host. Microbial phytases are sources from fungi, bacteria, and yeast; microfloral phytases are either plant or gut associated with intracellular or extracellular location (Rao et al. 2009). The hydrolysis of phosphates by phytases is carried out by the six phosphate groups that generate products that become substrates for subsequent hydrolysis. Some authors have reported that majority of phytases cleaves five of the six phosphate groups at the same time (Konietzny and Greiner 2002). On the basis of the reaction that involves the initiation of dephosphorylation of the phytate molecule, phytases are classified as 3-phytases (EC 3.1.3.8), 4-phytases, or 6-phytases (EC 3.1.3.26), 5-phytases (3.1.3.72). Based on this classification, the presence of phytases can be related. 3-phytases are present in bacteria (*Methanobacterium thermophila*, *Bacillus* sp., and *Klebsiella*) and fungi (*Aspergillus niger*, *Pseudomonas*, *Cicer arietinum Cicer arietinum)* (Sajidan et al. 2004); 4-phytases and 6-phytases are extracted from *E. coli*, *Yersinia* sp., *A. niger*, *Peniphora lycii*, grains and oilseeds of higher plants (Barrientos et al. 1994); and 5-phytases are found in *Selenomonas* sp. and lily pollen (Puhl et al. 2008). Few phytases extracted from bacteria and fungi are highly specific for PA while others exhibit broad substrate specificity by hydrolyzing structures such as ATP, ADP, glucose-6-phosphate, and phenyl phosphate that are dissimilar to PA.

Other classification includes on the basis of their catalytic activity, position of the hydrolysis initiation site of phytate molecule, and protein motifs. On the basis of catalytic machinery, phytases are subdivided into four groups such as β-propeller phytases (BPP) (E.C.3.1.3.8), protein tyrosine phosphatase-like phytases (PTP), purple

acid phytases (PAP) (E.C.3.1.3.2), and histidine acid phosphate (HAP) (E.C.3.1.3.2) (Mullaney and Ullah 2003). Among these, BPPs have a strong stability under alkaline conditions whereas the others have acidic pH optima (Tye et al. 2002). BPPs show 90–98% sequence identity among themselves revealing conserved motifs that are characteristically pursued by researchers. HAPs, on the other hand, share a catalytic N terminal RHGXRXP motif and C terminal HD motif that constitute the active site that favor a histidine involved nucleophilic assault in the active site followed by hydrolysis of phosphohistidine intermediate (Vincent et al. 1992). An overall reaction of phytases on phytic acid is given in Fig. 13.1a, b. Presently, the activation of phytases is undertaken by the consumers utilizing production or preliminary treatment procedures that are expensive, troublesome, and/or time-consuming. Phytases from microbial origins are now considered with great potential for development of phytic acid inhibited grains or plant sources ready for human consumption. Strains of microbial origins such as bacteria and fungi have been banked on for production of phytase. Bacteria from soil have been confirmed for phytase production by

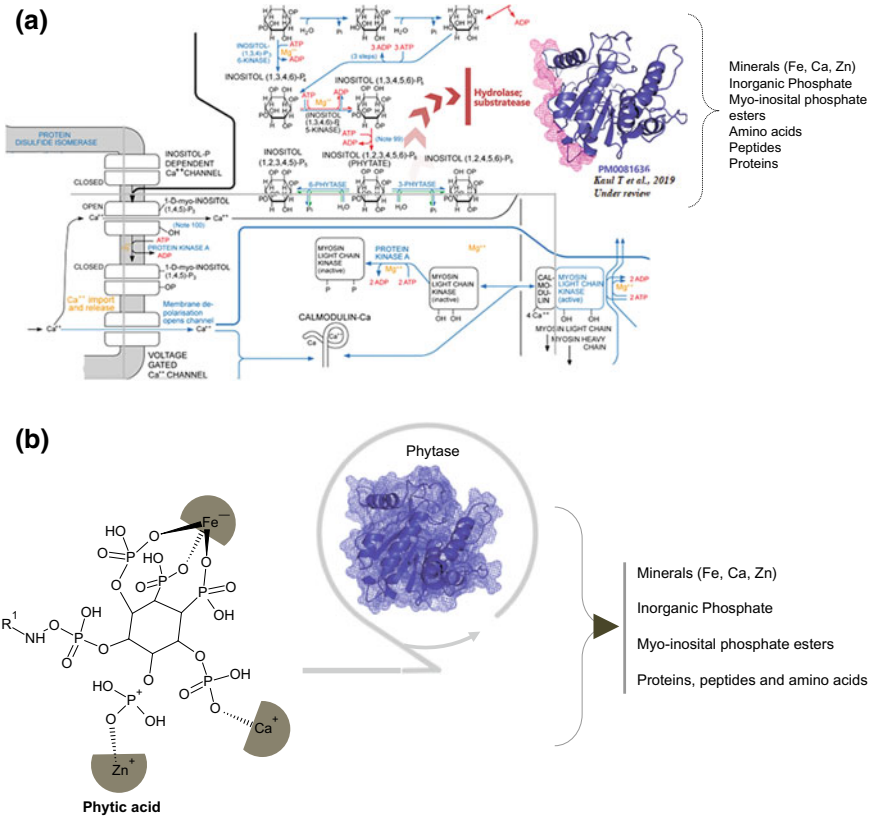

Fig. 13.1 **a** The resulting products after the action of phytase on phytic acid. **b** Reaction mechanism of phytic acids response to phytase

screening on phytase screening medium (Singh et al. 2013) and genera such as *Klebsiella*, *Bacillus,* and *Pseudomonas* have also been identified (Griener and Carlsson 2006). Among the ~230 fungal isolates screened, belonging to *Rhizopus*, *Penicillium*, *Mucor,* and *Aspergillus*, extracellular phytase production was confirmed to be most active in *A. niger* (Shieh and Ware 1968), *Aspergillus ficuum*, followed by *Aspergillus oryzae*, *Aspergillus flavus,* and *Aspergillus repen* (Howson and Davis 1983).

13.6 Phytases and Their Ideal Transgenic Plant Expression

An initial validation of the phytases that showed high activity for hydrolysis of phosphorus among early studies showed specificity for the phytate as the only source of phosphorus was ensured. Irving and Cosgrove (1972) validate the HAP from *A. niger* (PhyA) as a candidate of interest as it showed a bimodal pH profile at 2 and 5.5. Such studies laid the foundation for recombinant DNA technology that ensured the cloning and overexpression of such phytases for commercialization and review (Mullaney et al. 1991; Konietzny and Greiner 2002; Rao et al. 2009; Yao et al. 2012; Lei et al. 2013; Fan et al. 2013). Such ease in overexpression strategies shifted the studies from bulk production to application of phytases according to their biophysical and biochemical efficiency such as proteolytic tolerance, heat, and pH stability with catalytic efficiency. AppA phytases from *E. coli*, *P. lycii,* and *A. niger* (AnPhyB) belonging to HAPs have been pursued for commercialization as their pH profile is compatible in many animal models. Interestingly, comparative studies reveal *E. coli* phytases are less thermostable than fungal equivalents. Therefore, the search for phytase has now exploded that starts with mining of new phytases for biotechnological applications that reveal in-depth information of their enzymatic potential and structure. In accordance with many reviews (Rao et al. 2009; Li et al. 2010; Yao et al. 2012) and in view of the search for ideal phytase, sources for phytases have been extended to extreme environments such as glaciers (Huang et al. 2009) and thermophilic fungi (Singh and Satyanarayana 2011). The integration of bioinformatics in the field of phytases has made an impression with the recognition of fingerprint motifs and the increased number of phytase gene sequences in public databases (Fan et al. 2013).

Three approaches are attempted by researchers to reduce the PA levels in grains and seeds as a means to biofortification. These include generation of transgenic plants by manipulations of the PA biosynthetic pathway, expression of phytases in the edible portions of transgenic plants, and the use of conventional breeding approaches to induce mutations in low PA mutants. Alleviation of phytate levels in crops has been a major success using the strategy of recombinant microbial phytase in the edible parts of transgenic plants. The edited or generated transgenic plants serve as bioreactors for the production of phytases that can be bio-farmed for improving phytoremediation and plant phosphorus acquisition in a cost-effective approach. Such transgenic approaches favor the use of microbial phytases than plant phytases as the latter are relatively less thermostable and have low efficacy (Reddy et al. 2013).

Genetically modified low phytic acid plants could be a novel contribution to the reduction of micronutrient malnutrition and animal waste phosphorus. Nevertheless, additional research needs to be done to understand the molecular biology and genetics of phytic acid accumulation during seed development, the negative and positive roles of dietary phytic acid in human health, and the feasibility and effectiveness of the sustainable implementation of this approach at the community level. The TILLING population was developed by random mutations using ethyl methanesulfonate (EMS) chemical mutagen agents for generation of low phytic acid content as well as high endogenous phytase activity showing mutants in Pusa-Basmati rice (Shukla and Singh 2012). RNAi technology has been used to reduce maize phytic acid by silencing MRP4 ATP-binding cassette (ABC) transporter (Shi et al. 2007; Gupta et al. 2011). In diets, based on unrefined cereals or legumes, the bioavailability of several micronutrients, such as Ca, Fe, Zn, I as well as vitamins can be quite low, due to "high phytic acid" (PA or phytate)—an antinutrient chelator of minerals, micronutrients, proteins, starch, and vitamins causing metabolic disorders related to these nutritional factors. Transgenic tomatoes expressing the *Bacillus* phytase gene have been successfully developed. T_4 lines have been validated via molecular analyses and feed trials in mice [Kaul et al. under review, 2019; Patent File No: 201711038417 (TEMP/E-1/39247/2017-DEL)]. We visualize that our phytase-rich tomatoes with enhanced antioxidant content (beta-carotene, lycopene); when consumed raw shall serve as a first commercially available product that can be targeted to developing countries with huge populations surviving on monotonous plant-based diets containing high PA or phytate content with no or low supplementation of dairy or animal products.

In the past ten years, combined efforts of breeding programs and plant genetics have resulted in new variants of rice, barley, and maize that produce very low amounts of phytic acid and normal amounts to total phosphorus. These mutants are largely a result of single genes with recessive alleles that encode or contribute to phytic acid synthesis. Further improvements have been observed in breeding new cultivars with high yields when grown under optimal to nominal conditions. A wave of new techniques has now distanced the use of classical genetics and standard plant breeding methods. The resultant low-phytate cultivars and hybrids have been recognized widely for their contribution in balancing the nutritional requirement of the world in spite of the current global debate on transgenics.

13.7 Conclusion

For optimum use of the beneficial phytate activities in the gut, phytate, on the one hand, has to be degraded to avoid inhibitory effects on the intestinal mineral absorption. On the other hand, if anticancer, ant oxidative, and anticalcification activities of phytate are to be used, any phytate hydrolysis would be counterproductive. Thus, the actual demand of a population to either improve mineral and trace element bioavailability or to help prevent cancer, kidney stone formation or other civilization diseases,

will decide whether or not phytate will be welcome in our daily diet. It is important that researchers continue both to utilize existing resources and to explore the new genomic tools (VandenBosch and Stacey 2003) for modifying the phytate content of seeds. Undoubtedly, any potential negative consequences to plant growth and development will need to be evaluated and monitored in newly developed plant lines. When indirect consequences do arise, however, they should be viewed merely as additional challenges to be overcome as we strive to develop a more nutritious food supply for a growing world.

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Chapter 14
Biofortification in Pearl Millet: From Conception to Dissemination

Alphonse Vinoth and Ramalingam Ravindhran

Abstract Biofortification is an economical and sustainable process of delivering essential micronutrients through staple crops. The biofortified crops developed by HarvestPlus through conventional breeding continue to reach the target populations of Asia and Africa in order to reduce the burden of iron, zinc and vitamin A deficiency. Pearl millet, a dryland crop of the arid and semi-arid tropics is a suitable crop for iron biofortification as it harbours sufficient genetic variability for grain iron (Fe) and zinc (Zn) in the existing germplasm. Zn is highly correlated with grain Fe and therefore enhanced as an associated trait during the breeding for high-iron pearl millet. ICTP 8203 Fe-10-2, an iron-biofortified pearl millet (Fe-PM) variety developed via intra-population improvement of *iniadi* germplasm, was commercially released for cultivation in Maharashtra, India, by 2014. Efficacy trials undertaken in women and children feeding on Fe-PM meals revealed an enhancement in their micronutrient status as well as their functional outcomes. Disbursement of Fe-PM through public–private seed markets worked out to be cost-effective. Farmers readily adopted Fe-PM for cultivation based on its superior agronomic performance rather than the preference for consumer attributes. On the other hand, consumers expressed their willingness to pay for Fe-PM over regular pearl millet because of its favourable sensory characteristics. Therefore, investment on high-Fe hybrids would bridge the gap between the farmers and consumers acceptance of biofortified millets. Iron biofortification is also limited by the presence of antinutrients like phytates and polyphenols as they hinder the Fe bioavailability. The development of biofortified crops with reduced antinutrients needs careful evaluation as they have a significant role in protection against diseases and seedling growth. This review paper deliberately describes the success of high-Fe pearl millet in India and the lessons to be learnt for expanding the biofortification efforts to other small millets.

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Keywords Biofortification · Iron deficiency · HarvestPlus · Pearl millet · Conventional breeding · ICRISAT · Dhanshakti · Seed distribution system

Abbreviations

AAS	Atomic absorption spectroscopy
CGIAR	Consultative Group on International Agricultural Research
DALY	Disability-adjusted life year
EAR	Estimated average requirement
EEG	Electroencephalography
Fe	Iron
Fe-PM	Iron-biofortified pearl millet
G × E	Genotype-by-environment
Hb	Haemoglobin
ICP-OES	Inductively coupled plasma optical emission spectroscopy
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
Lpa	Low-phytate mutants
NSSO	National Sample Survey Office
OPV	Open-pollinated variety
PA	Phytic acid
SAU	State Agricultural University
UNICEF	United Nations International Children's Emergency Fund
XRF	X-ray fluorescence spectroscopy
Zn	Zinc

14.1 Introduction

Micronutrients are vital components of human nutrition predominantly obtained from plant-based foods. Plant dietary sources like fruits and vegetables provide high amounts of micronutrients. However, majority of the worlds' population inhabiting developing countries cannot afford for diverse diet and instead rely solely on staple crops (rice, wheat, millet, cassava and maize) containing low levels of micronutrients. The nutritional insecurity prevailing worldwide over the years has bloomed into a serious global challenge of the humankind, rightly termed as hidden hunger (Welch and Graham 1999; Graham et al. 2001; Bouis et al. 2011). The need for immediate attention to curtail micronutrient malnutrition emerged from the estimates testifying 12 million low-birth-weight births per year and malnourishment in around 162 million preschool children (Copenhagen Consensus 2004; http://www.copenhagenconsensus.com). Another statistical survey cited iron (Fe) deficiency

afflicting approximately 2.7 billion people (Hirschi 2009). Micronutrient malnutrition increases the mortality and morbidity rates, especially in children and pregnant women (Welch and Graham 1999). In order to overcome the widespread micronutrient malnutrition, several countries adopted fortification of processed foods, but met with limited success owing to the lack of industrial agriculture, food processing and distribution networks (Pfeiffer and McClafferty 2007). This scenario led to the advent of an alternative strategy called biofortification.

Plant-based food biofortification programme was launched in 2004 by HarvestPlus (for detailed history, visit www.harvestplus.org) with the goal of reducing micronutrient malnutrition in Asia and Africa. It was envisioned as a sustainable and cost-effective approach for better nutrition amongst the rural poor who cannot afford commercially processed fortified foods (Bouis 1999; Meenakshi et al. 2010; Hotz 2013). Biofortification is an agricultural strategy that aims to develop crops with higher micronutrient concentration and bioavailability in their edible tissues (White and Broadley 2005; Nestel et al. 2006; Mayer et al. 2008). Three major nutrients (iron, zinc and vitamin A) were the targets to be increased upon in edible parts of seven staple food crops (rice, beans, cassava, maize, sweet potatoes, pearl millet and wheat) (Carvalho and Vasconcelos 2013). HarvestPlus preferred conventional breeding techniques over genetic engineering to biofortify staple crops as the former had widespread public acceptance and a simple legal framework (Bouis 2000; Hirschi 2009; Winkler 2011). The exploitation of sufficient genetic variation for grain nutrient density from the germplasm collections of seven staples resulted in the production of biofortified crops through a multistage breeding process (Fig. 14.1).

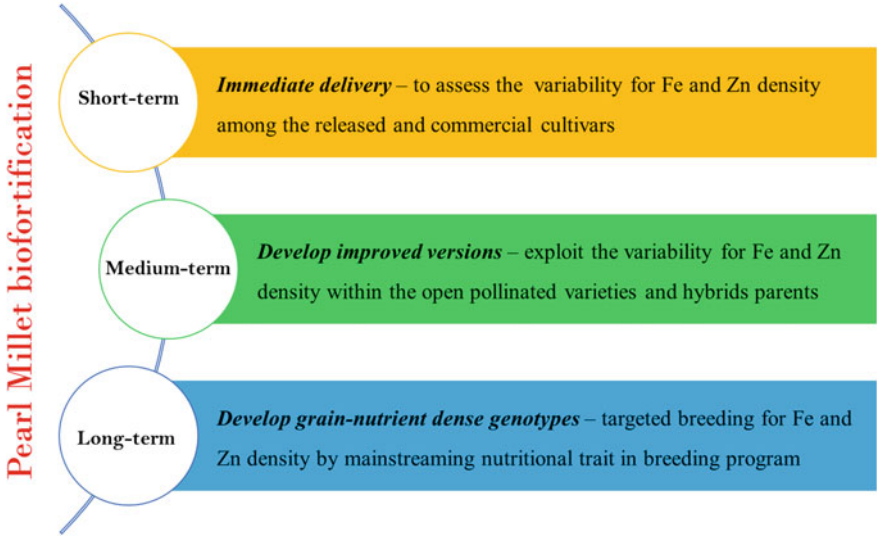

Fig. 14.1 Objectives of biofortification programme by HarvestPlus to develop high-iron pearl millet

14.2 Iron Deficiency—Global Status and Health Consequences

Iron (Fe) is the key functional component of haemoglobin (Hb), responsible for oxygen transport to body tissues through the blood. Fe is also important for the proper functioning of cardiac and skeletal muscles and is critical for brain development in children (Gangashetty et al. 2016). The recommended dietary allowance of Fe is high for women (15 mg/day) than men and children (10 mg/day) (FAO 2003; ICMR 2009). Lack of Fe below the recommended levels (<12 g/dl of Hb) leads to iron deficiency anaemia, which is considerably found in one-third of the world's population (Boccio and Iyengar 2003). The United Nations International Children's Emergency Fund report states the occurrence of Fe deficiency in 67% of children in Africa (UNICEF 2004). In India, about 70% of the children below 3 years and women of reproductive age suffer from anaemia (Krishnaswamy 2009). Iron deficiency anaemia limits the work efficiency of adults thus bringing a severe setback for the economic development of a nation (Shivran 2016). Fe deficiency results from haemorrhage, increased demand due to diseases and inadequate intake/bioavailability from diet (Rosegrant and Cline 2003; Lemke 2005; Skalicky et al. 2006), of which the latter which is predominant in developing countries affects the foetal brain development and diminishes the immunity, physical growth and cognitive abilities of children (Caballero 2002).

14.3 Pearl Millet—The Crop Vehicle for Iron Biofortification in India

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a staple cereal of rural populations (~0.2 billion) inhabiting the arid and semi-arid tropics of central and southern India and sub-Saharan Africa. It is the sixth most important cereal reaching a global annual production of 20 million metric tons (Gangashetty et al. 2016). Africa (countries spreading over 7000 km from Senegal to Somalia) and India contribute an equal share to worlds' pearl millet production. It was domesticated as early as 4000 years ago in Africa which then spread to India and attained the status of much-favoured staple food grain, feed and fodder crop in drought-prone areas (Gangashetty et al. 2016). In India, Gujarat, Maharashtra and Rajasthan are the major pearl millet-growing states, altogether accounting for 70% of production and the highest concentration of consumers. According to National Sample Survey Office (NSSO) Report No. 508, pearl millet is consumed by more than 90% of rural and urban populations belonging to the low- and middle-income groups (Basavaraj et al. 2010). Pearl millet is highly preferred for cultivation by marginal farmers on unirrigated lands owing to its adaptability to tolerate heat and high productivity under conditions of poor soil fertility and low-moisture content (Gupta et al. 2015). Apart from its characteristic resistance to abiotic stress, pearl millet grains are highly nutritious with a balanced

amino acid profile, low glycemic index and gluten-free (Andrews and Kumar 1992; Dahlberg et al. 2004; Sehgal et al. 2004). Considering the nutrient richness, high productivity under harsh environmental conditions and utilization by low-income people, HarvestPlus recommended pearl millet as the crop for iron biofortification in India, while West Africa remained the choice of a secondary country. Iron biofortification was the primary trait of focus in pearl millet with zinc (Zn) as an associated trait. The breeding target levels for iron was set up at 77 μg/g, an increment of about 30 μg/g from the baseline.

14.4 Conventional Breeding Process to Develop High-Iron Pearl Millet

14.4.1 Phase I (Discovery, 2003–2008)

Breeding of crops for any desirable trait involves the selection of parental material as a prerequisite. Elite parental lines are chosen based on the magnitude of genetic variability for the desirable trait in the germplasm pool, correlation with agro-morphological traits and the extent to which the trait is heritable. Genetic variability for grain nutrient content is influenced by agronomic practices, soil fertility, genotype and environment. The HarvestPlus project run by International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, initiated an intensive search for sources of higher grain Fe content in pearl millet by the year 2004 (Velu et al. 2007). The study carried out across two seasons identified the entries (developed largely from the *iniadi* germplasm known for its early maturity, large seed size and compact panicles) with high levels of grain Fe, almost 50% greater than that in maize. Most of these varieties exhibited a positive correlation between the Fe and Zn content indicating the likelihood of simultaneous genetic improvement for the elevated levels of both micronutrients. Another notable finding was the significant positive correlation of 1000-grain weight with Fe and Zn content per grain (Velu et al. 2007). In another study, Velu et al. (2008) screened 68 improved populations comprising of composites and open-pollinated varieties (OPVs) developed by ICRISAT for grain Fe and Zn content. The results from the experimental trials ranked ICTP 8203 amongst the top five with higher grain Fe (79.9 mg/kg) and Zn (47.1 mg/kg) content. ICTP 8203 is an OPV developed from *iniadi* landrace that was commercially released in 1988 and still under cultivation in India (Rai et al. 1990). Sufficient genetic variability recorded for grain nutrients amongst the S_1 progenies of ICTP 8203 suggested good prospects for intra-population improvement. Moreover, the progenies with high grain nutrients were the earliest to flower (Velu et al. 2008).

Many other investigations on pearl millet accessions also revealed no significant correlation between grain yield and Fe and Zn densities (Gupta et al. 2009; Govindaraj et al. 2009; Burger et al. 2014). This brought out the fact that breeding for grain nutrient density could be achieved without compromising on large grain size

and grain yield, the agronomic traits preferred by the farmers in pearl millet-growing regions of India and Africa. Heritability estimate is a useful measure in biofortification programmes as it determines the genetic variation amongst individuals in a population for a specific trait and the relative amount of heritable portion of such variation (Gomez-Becerra et al. 2010). High heritability for grain Fe and Zn (70–80% for both micronutrients) reported in pearl millet indicates the predominance of additive gene effects (Velu et al. 2007; Gupta et al. 2009; Govindaraj et al. 2011, 2013; Bashir et al. 2014; Kanatti et al. 2014).

14.4.2 Phase II (Development, 2009–2013)

Pearl millet biofortification for high-Fe content was focussed on three objectives to identify and exploit suitable genetic material (Fig. 14.1). Conventional breeding methods commonly employed for varietal improvement in pearl millet include population improvement approaches, mass selection and marker-assisted selection. The population improvement approach was favoured by public and private sector companies as there was sufficient genetic variability for grain Fe and Zn in breeding lines, populations and hybrid parents. As large intra-population variability for Fe and Zn densities existed in ICTP 8203, seven improved progenies of the same cultivar were developed between 2009 and 2010 (Rai et al. 2013). Genotype-by-environment (G × E) interaction complicates the breeding process as it reduces the overall genetic gains of desired traits (Shafii and Price 1998). In pearl millet biofortification programme, multilocation trials were deemed mandatory as significant G × E interaction effect for grain Fe and Zn densities was recorded (Gupta et al. 2009; Velu et al. 2011; Govindaraj et al. 2013; Burger et al. 2014). On-farm trials of four progenies of ICTP 8203 out of seven from the previous study by Rai et al. (2013) during 2010 and 2011 across India identified ICTP 8203 Fe-10-2 to contain 9% more Fe and 11% greater yield than the parental line. This improved version of a commercial OPV containing 100% of the iron target was officially released as the first biofortified crop (ICTP 8203-Fe; designated hereafter as Fe-PM) in Maharashtra, India, by 2013. Later, it was designated as "Dhanshakti" and released for cultivation by February 2014 in all pearl millet-growing states of India.

Concurrently, a high-Fe version (ICMV 221 Fe-11-1) of another OPV was developed by intra-population selection for Fe density (Rai et al. 2013). In view of the development of biofortified hybrids as the prime focus of pearl millet biofortification programme, multilocation field trials of hybrids were conducted during 2011 and 2012. An experimental hybrid (ICMH 1201) had Fe and Zn density comparable to ICTP 8203 but with 38% higher yield (Rai et al. 2013). Breeding lines, hybrid parents, improved populations and composites of pearl millet bred for grain nutrient density were largely developed from *iniadi* germplasm. A search for accessions with still higher Fe and Zn than Fe-PM was initiated with 297 accessions from Togo, Ghana and Burkina Faso. Of these, 27 accessions had Fe and Zn density in the range of 95–121 mg/kg and 59–87 mg/kg, respectively (Rai et al. 2014). Therefore, the

prospects of developing high-yielding hybrids with higher levels of Fe and Zn densities loom high using *iniadi* germplasm as the parental breeding lines.

14.5 Analytical Tools to Measure Iron and Zinc

Measurement of trace micronutrients in grains is an important aspect of biofortification programme during the breeding process. Various analytical techniques that accurately determine the levels of micronutrients, especially Fe and Zn in biofortified crops, include inductively coupled plasma optical emission spectroscopy (ICP-OES), atomic absorption spectroscopy (AAS), colorimetric staining and X-ray fluorescence spectroscopy (XRF). Amongst these tools, ICP-OES has high accuracy and sensitivity with detection limits of μg/kg and therefore considered to be the "gold standard" for micronutrient analysis in plant samples. AAS and ICP-OES require sample pre-processing which is laborious and expensive, while colorimetric staining is time-consuming, and therefore, the above tools are recommended only for a limited number of samples. XRF has recently been employed by HarvestPlus for micronutrient analysis in whole grains and flour. XRF is the ideally suited analytical instrument for micronutrient breeding programmes as it is cheaper, easy to operate, facilitates large-scale screening and does not require pre-processing of samples. Two XRF instruments, namely Oxford Instruments X-Supreme 8000 and the Bruker S2 Ranger installed by HarvestPlus can process around 100–200 samples per day. XRF and ICP-OES thus complement each other in selecting nutrient-dense lines during various stages of the breeding programme (Stangoulis and Guild 2014).

14.6 Efficacy Trials and Nutrition Evidence

Efficacy trials determine the nutritional impact of biofortification in humans who consume biofortified foods by a randomized, controlled experimental study design (Haas 2014). The potential of biofortified crops in curbing the hidden hunger could be truly realized by their micronutrient bioavailability. Bioavailability is defined as the amount of nutrient that fills the gap between intake and daily requirement; in nutrition terms, it refers to the nutrients in a food that is absorbable and utilizable for body metabolic processes. The breeding target set up by HarvestPlus for preschool children (4–6 years old) and non-pregnant, non-lactating women of reproductive age were that the incremental amount of iron and zinc in biofortified pearl millet could provide approximately 30 and 40% of the Estimated Average Requirement (EAR), respectively. Consequently, the bioavailability of additional iron and zinc (assumed to be 5% for Fe and 25% for Zn) from the biofortified pearl millet determines the actual improvement in micronutrient status of deficient populations (Saltzman et al. 2013).

A study was undertaken by Cercamondi et al. (2013) amongst the young Beninese women of southern Nigeria to ascertain the use of iron-biofortified pearl millet (Fe-PM) as a promising approach to combat iron deficiency by comparing its efficacy against regular-iron pearl millet (DG-9444) and postharvest iron-fortified pearl millet. The iron bioavailability indicators, namely fractional iron absorption and total iron absorbed, were investigated from the blood samples after a 5-d composite meal design by stable isotope technique (for detailed study design, refer Cercamondi et al. 2013). The data obtained from 20 women at baseline, during the feeding series and endpoint revealed an increased absorption of total iron (~2 times) from Fe-PM meals compared with regular-iron millet meals. This clearly points to the increased bioavailability of additional iron (~3.5 times) from Fe-PM, despite the fact that it contained higher phytic acid (PA) concentrations (~2 mg/g) than the regular-iron millet. The increase in the concentration of PA was, however, counterbalanced by the reduced molar ratio of PA:iron, plummeting the chelating activity of PA (Cercamondi et al. 2013). Concurrently, another team lead by K. Michael Hambidge, Section of Nutrition, Department of Pediatrics, University of Colorado Denver, headed towards India to study the efficacy of Fe-PM (Kodkany et al. 2013). The feeding trial comprising of young children aged 2 years revealed that the absorption of Fe and Zn from Fe-PM test meals like sheera, uppama and roti exceeded the EAR for this age group. As observed in the previous study, enhanced concentrations of Fe and Zn in pearl millet resulting from biofortification efforts was bioavailable due to the decreased molar ratio of phytate:Fe/Zn in the biofortified grain (Kodkany et al. 2013).

HarvestPlus not only visualized biofortified staple crops as a complementary intervention to enhance the micronutrient status but also to improve the functional outcomes of deficient populations in terms of cognitive performance and physical and brain activity. The affiliates of the HarvestPlus gathered at the American Society of Nutrition Scientific Sessions and Annual Meeting at Experimental Biology 2014 in San Diego, CA, for the symposium "Are Biofortified Staple Food Crops Improving Vitamin A and Iron Status in Women and Children?" and discussed the preliminary findings from six efficacy trials and two effectiveness trials (De Moura et al. 2014). The total body iron status directly impacts the work efficiency by regulating the physical performance (Haas and Brownlie 2001). J. D. Haas and S. V. Luna recorded significant improvement in the network efficiency of Indian adolescent boys and girls after 6 months of consuming Fe-PM. The subsamples of participants from the former study conducted by Haas (2014) were also evaluated for perceptual and cognitive performance and associated brain dynamics using electroencephalography (EEG). Surprisingly, Drs. Murray-Kolb and Wenger documented changes in both behaviour and brain dynamics as a response to the variation in iron status of individuals feeding on Fe-PM. Likewise, Finklestein et al. (2015) also disclosed the influence of Fe-PM in resolving the iron deficiency amongst the school-aged children (12–16 years). The results of the above studies were comparable to iron fortification and supplementation trials and thus strongly prove that biofortified millets could guarantee to reduce the burden of micronutrient deficiency in poor, rural-based families of Asia and Africa who consume millets as the primary food staple.

14.7 Delivery Models, Consumer Acceptance and Cost-Effectiveness

The effectiveness of biofortification can be measured only by population-based studies since the product development alone cannot bring out the end results. The factors that influence increased consumption of biofortified crops include effective dissemination and marketing, farmers demand, consumer education and acceptance and intervention implementation costs. HarvestPlus has taken advantage of the well-established seed distribution system in India, and this route would more likely facilitate wide dissemination of Fe-PM at limited costs. The partnership of HarvestPlus with Nirmal Seeds Ltd., and Shakti Vardhak, the leading commercial entities in pearl millet seed sales, to market and deliver Fe-PM is a sustainable strategy for the future. Presently, HarvestPlus encourages the development of high-yielding, high-iron hybrids with stable yield and iron performance for the different agro-ecological zones in India by partnering with five State Agricultural Universities (SAUs) and 15 seed companies, most notably Nirmal Seeds and Tempest India. The SAUs and seed companies expedite genotype-by-environment (G × E) testing of hybrids and inbred lines developed at ICRISAT to raise their own commercial high-iron hybrids, for which the iron content is measured free of charge by HarvestPlus (Cherian 2014; Rai 2014). This initiative has set a target to reach at least 1.5 million farming households by the end of 2018 through the private and public sector seed companies. Nonetheless, the private seed companies must be well supported by the Consultative Group on International Agricultural Research (CGIAR) centre to maintain the genetic purity of parental lines.

Farmers tend to readily adopt Fe-PM based on its agronomic performances like drought tolerance, resistance to downy mildew and end-use quality traits. It is always worthy investigating the acceptance and valuation of a biofortified staple food crop by farmers before its commercialization. In this context, a study was undertaken by Birol et al. (2011) during the development phase of Fe-PM to understand the farmers' appraisal of various consumptions and production attributes of pearl millet seeds. The straightforward recommendation from the study was the emergence of trade-offs between nutritional traits and yield. The consumers and the sale producers differ in their preference for traits, and therefore, hybrid varieties of Fe-PM need to be raised to cover a large number of target groups (Birol et al. 2011).

Consumer acceptance plays a crucial role in the widespread consumption of biofortified foods by target populations. As the iron enrichment in pearl millet is an invisible trait, consumers' acceptance is expected to be quite high and it would be preferred over conventional pearl millet based on other sensory traits such as shape, size, texture, colour, odour and cooking qualities (Birol et al. 2015; Huey et al. 2017). Various other aspects that reflect the consumer acceptance are their willingness to pay for biofortified varieties over conventional varieties, nutritional information, branding and the nature of agency that certifies and delivers the biofortified crops. A study was undertaken by Banerji et al. (2016) to evaluate the rural consumers' preference for Fe-PM grains and bhakri (a thick flatbread) in three districts

of Maharashtra, India. This is the first consumer acceptance study for a mineral-biofortified crop that helped us to understand people's preference for Fe-PM over the conventional varieties despite the lack of visual differences. The study revealed that the consumers were willing to pay a substantial premium for Fe-PM even without the nutrition information owing to their favourable sensory characteristics. Also, certification, branding and promotion of Fe-PM through international agencies resulted in higher adoption and consumption rates (Banerji et al. 2016).

Cost-effectiveness analysis is another important criterion to ascertain if the intervention is economically viable and sustainable in developing countries. The effectiveness of biofortified crops was measured based on the cost-to-benefit ratio in the early years (Bouis 2003), while recently the saving of disability-adjusted life years (DALYs) is considered to be a significant measure of biofortification success (Stein et al. 2005; Meenakshi et al. 2010). The effectiveness studies so far were focussed upon orange sweet potato and maize, as a visual change in these biofortified foods required greater public sensitization. A large-scale multidisciplinary effectiveness study on the delivery models of vitamin A-biofortified sweet potato in African countries has revealed the biofortification intervention for human nutrition to cost about $15–20 per DALY averted, thus working out to be cost-effective by World Bank standards (De Moura et al. 2014). Likewise, the use of Fe-PM is expected to cost $US 2–20 per DALY in Africa (White and Broadley 2009), which is economically viable compared to dietary diversification, supplementation or food fortification programmes. A nationwide community sensitization on the need for adoption, diffusion and consumption of Fe-PM by poor populations is the need of the hour to reap the fullest benefits of nutrition from biofortified millets.

14.8 Antinutrients—Hindrance to Fe and Zn Bioavailability and Ways to Deal with It

Antinutrients are dietary inhibitors that chelate essential mineral elements thereby lowering their bioavailability. Antinutrients include phytates, tannins, polyphenols and dietary fibre (Gibson et al. 1994; Liu et al. 2006; Hambidge et al. 2010). The proportion of Fe and Zn absorbed from plant-based diets in humans correspond to 5 and 25%, respectively (Hotz and McClafferty 2007). The bioavailability of Fe and Zn is prominently affected by the presence of phytate in cereal grains when the molar ratio of phytate/Fe and phytate/Zn exceeds above 1 and 6, respectively (Lönnerdal 2002; Hurrell 2003). The dose-response inhibitory effect of phytate is gradual for Zn while being acute for Fe bioavailability. Similarly, polyphenolic compounds strongly inhibit iron bioavailability as reported in pearl millet by Lestienne et al. (2005).

Intra-specific genetic variation for grain phytate concentration has been recorded in pearl millet (Abdalla et al. 1998). Raboy (2003, 2007) reported both natural and induced low-phytate mutants (*lpa*) in rice, maize, wheat, barley and soybean. Consumption of these *lpa* mutants over conventional cereals improved the mineral

nutrition in humans (Adams et al. 2002; Mendoza 2002). Moreover, a weak correlation between grain phytate and mineral concentration (Cichy et al. 2005; Reddy et al. 2005) and non-overlapping of QTLs controlling these traits (Stangoulis et al. 2007; Waters and Grusak 2008) suggest the possibility to breed for nutrient-dense crops with low-phytate concentrations. Breeding for high iron in staples also resulted in increased concentrations of Fe absorption inhibitor, polyphenols (Tako et al. 2014, 2015). Fe-PM had elevated levels of 15 unique parent polyphenolic aglycones that inhibited Fe absorption in in vitro/*Caco-2* cell and *Gallus gallus* models. Therefore, the increased Fe content in Fe-PM did not lead to a proportional increase in serum ferritin (Tako et al. 2015).

Phytates and polyphenols, though being designated as antinutrients in crops, from the nutritionists' point of view, they are essential for protection against chronic diseases (Vucenik and Shamsuddin 2003, 2006; Zern and Fernandez 2005). From the agricultural perspective, reduced levels of these compounds lower the yield performance and decrease the resistance to pests (Bregitzer and Raboy 2006). Therefore, enhancement of mineral bioavailability in crops by reduction in the levels of antinutrients complicates the process of biofortification, as any such modifications have certain implications on public health and agronomic performance. Various questions arising in this aspect could only be addressed when long-term intervention studies are undertaken with low-phytate staple foods.

14.9 Conclusions and Future Challenges

The journey to biofortify high-iron pearl millet has traversed fifteen long years till date since its inception. The dissemination of biofortified pearl millet across the nation is being realized by mainstreaming of the grain Fe trait into the breeding process. The first-wave release of Fe-PM (ICTP 8203-Fe), an OPV, was limited in its potential to expand as 95% of the area under cultivation in India is planted to hybrids. The second-wave hybrid cultivars commercialized in 2014 produced 41% greater grain yield than ICTP 8203-Fe but still unable to reach the target iron level set up by HarvestPlus (>77 ppm; >100% target). This can be attributed to the environmental influence on grain nutrient density which is poorly understood. A broader partnership at the national level must be bridged between the public and private sectors to generate multi-environment data on grain nutrient density in pearl millet.

Distribution of Fe-PM seeds is equally important to reach the target populations on a large scale. It is highly recommended to exploit the institutional government programmes, such as subsidized public food distribution system and school midday meal programmes for the delivery of Fe-PM grains and value-added products. Another potential hindrance to improve Fe status in target populations using Fe-PM is the abundance of phytates. As breeding for low-phytate pearl millet is not immediately attainable, novel transgenic, RNA silencing and genome engineering approaches targeting phytic acid biosynthetic genes could be employed. Henceforth, a one-time investment on the production of region-specific hybrid varieties with up

to 100% of the iron target, reduced antinutrient factors and superior yield performance and cost-effective delivery models would drive forward the HarvestPlus goal of reducing iron deficiency in Asia and Africa.

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Chapter 15
Biotechnology for Nutritional and Associated Processing Quality Improvement in Potato

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Abstract Plant products comprise majority of human food intake globally. Therefore, it is expected that better nutritional availability for human beings can be achieved by nutritional improvement of food crops. Having achieved the food sufficiency in almost all parts of the world provides us the leverage to have a paradigm shift from "quantity increase" to "quality improvement" of crops. Improvement of quality will be crucial in future; it is almost sure that there will be significant reduction in land available for agriculture as compared to that available today. This means that in the lesser agricultural land, we shall have to produce more food in terms of nutrients. Potato (*Solanum tuberosum* L.) is the world's third most important crop in terms of human consumption. It is consumed in all countries of the world whether developing or developed and has been used as a primary nutritional source in many diets and as the basis for a variety of processed products. Ability of potato to produce highest nutrition and dry matter on per unit area and time basis, among major food crops, made FAO to declare it the crop to address future global food security and poverty alleviation during 2008. Although potato is a rich source of several nutrients such as protein, vitamin C, vitamin B6, and niacin, there is ample scope for improving its nutritional quality and making it more nutritious food. Worldwide conventional breeding technologies have given the mankind a large number of varieties having improved traits as compared to their predecessors. It has led to the development of a large numbers of cultivars of various crop plants which in turn has resulted in tremendous increase in their productivity. Recent advancements in the field of agricultural biotechnology have created a new domain to complement the methods of plant breeding. These biotechnological approaches are also being used for improving the nutritional quality as well as the processing attributes of potato. Using biotechnological tools, a large number of nutrients have been improved in potato. These include phenolics, vitamins, essential amino acids, protein, carbohydrates, and minerals. Certain anti-nutritional factors have been reduced in certain potato cultivars.

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Processing attributes of potato have also been improved using biotechnological tools such as gene silencing and gene editing. Recent improvement in genome-editing technologies is expected to further assist plant researchers to develop nutrient-rich potatoes in more targeted manner.

Keywords Genome editing · Essential amino acid · Phenolics · Resistant starch · Cold-induced sweetening · Protein · Anti-nutritional factors

15.1 Introduction

Universally, most of the past agricultural research programs and innovations have been predominantly focused on increasing productivity of crops. In other words, "quantity" has been the center around which majority of the crop improvement and relevant resource management activities have been formulated and executed. There is no doubt that this has been the requirement of the past times where producing and making food accessible to every human being was globally the top-most priority. Having achieved the food sufficiency in almost all parts of the world provides us the leverage to have a paradigm shift from "quantity increase" to "quality improvement" of crops. Improvement of quality also becomes essential as in future, it is expected that there will be significant reduction in land available to agriculture as compared to that is available today. This suggests that in the less land availability for agriculture, we shall have to produce higher nutrients.

Food has been viewed as a source to supply amount of nutrients just sufficient for survival and normal growth. However, since evolution, human desires have always been to strive for continuous improvements in terms of physical as well as mental strengths and longevity. Fulfilling these desires definitely necessitates looking into our food components and significantly improving it in terms of their (food components) nutritional quality. Agricultural innovation has always involved new, science-based products and processes that have contributed reliable methods for increasing productivity and sustainability. Biotechnology has introduced a new dimension to such innovations, offering efficient and cost-effective means to produce a diverse array of novel, value-added products and tools.

Globally, plant products comprise the vast majority of human food intake, irrespective of location or financial status. In some cultures, either by design or default, plant-based nutrition actually comprises 100% of the diet. Therefore, it is to be expected that nutritional improvement can be achieved via modifications of staple crops. Further, it has been suggested that food components can influence physiological processes at all stages of life. For example, inverse relationships have been observed between carotenoid-rich foods and certain cancers (Botella-Pavia and Rodriguez-Conception 2006). Other nutrient-related correlations link dietary fat and fiber to the prevention of colon cancer, folate to the prevention of neural tube defects, calcium to the prevention of osteoporosis, antioxidant nutrients to the scavenging of reactive oxidant species and protection against oxidative damage of cells, etc.

Potato has a definite place in the diet and is associated with good nutrition and health. Potatoes are uniquely positioned to be a valuable source of dietary vitamins, minerals, and phytonutrients because of their high per capita consumption. In most of the developed world, potatoes are by far the most eaten vegetable. Because of this high consumption, the vitamin and phytonutrient contents of potato will have much more dietary relevance and impact than food eaten in sparse quantities. Potatoes yield more calories per acre than any other major crop, a criterion that becomes even more important in light of the planet's ever-increasing population, food shortages, price spikes, and the recent trend of utilizing farmland for other commercial purposes. Collectively, these facts emphasize the impact potatoes can have on global nutrition in the future. These facts imply that any significant improvement in nutritional quality of potato will have even more than significant impact on human health and nutrition. Here we have described the biotechnology-based approaches for improving the quality traits of potato.

Worldwide conventional breeding technologies have given the mankind a large number of varieties having improved traits as compared to their predecessors. It has led to the development of a large numbers of cultivars of various crop plants which in turn has resulted in tremendous increase in their productivity. In conventional breeding, progeny inherit genes for both desirable and undesirable traits from both parents. Desired characteristics are conserved, and undesirable ones are suppressed/eliminated by repeatedly selecting superior individuals from each generation to be the parents of the next. However, the long breeding cycles, high heterozygosities, lack of various degrees of preciseness in hybridization, low frequencies of desirable mutations, and limit of using the genetic resources of primary and secondary gene pool have made new varietal development highly resource-demanding. Recent advancements in the field of agricultural biotechnology have created a new domain to complement the methods of plant breeding. These biotechnological approaches are also being used/can be used for improving the nutritional quality as well as the processing attributes of potato.

15.2 Tuber Composition and Dietary Importance of Potato

Nutrition is the processes by which we take in and utilize food substances. Nutrition is essential for growth and development, health, and wellbeing. Essential nutrients include carbohydrate, protein, fat, vitamins, minerals, and electrolytes. Recommended dietary allowance (RDA) of these important nutrients has been defined worldwide and is revised/updated from time to time. RDA for these nutrients in India is presented in (Table 15.1). Potatoes are approximately 80% water and 20% solids, although it can vary widely from cultivar to cultivar (Fig. 15.1). Of the 20 g of solids in a 100 g tuber, about 17 g are carbohydrate and 2 g protein. In addition to carbohydrates and proteins, potatoes are a good source of many vitamins and minerals (Fig. 15.1). According to the USDA nutrient database, 100 g of potatoes contains 4% of the RDA calorie intake, 33% of the RDA of vitamin C, the most abundant vitamin

Table 15.1 Recommended dietary allowances for Indians

Nutrient	Man	Woman	Pregnant woman	Children (1–9 years)	Girls (10–17 years)	Boys (10–17 years)
Protein (g/d)	60	55	78	16.7–29.5	35–40	35–50
Calcium (mg/d)	600	600	1200	600	800	800
Iron (mg/d)	17	21	35	9–16	27	32
Vitamin A (b-carotene) (μg/d)	4800	4800	6400	3200–4800	4800	4800
Thiamin (mg/d)	1.2–1.7	1.0–1.4	1.6	0.5–0.8	1.0–1.2	1.1–1.5
Riboflavin (mg/d)	1.4–2.1	1.1–1.7	2.0	0.6–1.0	1.2–1.4	1.3–1.8
Niacin equivalent (mg/d)	16–21	12–16	18	8–13	13–14	15–17
Pyridoxin (mg/d)	2.0	2.0	2.5	0.9–1.6	1.6–2.0	1.6–2.0
Ascorbic acid (mg/d)	40	40	60	40	40	40
Dietary folate (μg/d)	200	200	500	80–120	140–200	140–200
Vit. B12 (μg/d)	1	1	1.2	0.2–1.0	0.2–1.0	0.2–1.0
Magnesium (mg/d)	340	310	310	50–100	160–235	120–195
Zinc (mg/d)	12	10	12	5–8	9–12	9–12

Adapted from National Institute of Nutrition (2011)

in potatoes and 12% of the RDA for potassium. Also, potato tubers contain an array of other small molecules, many of which are phytonutrients. These include polyphenols, flavonols, anthocyanins, phenolic compounds, carotenoids, polyamines, and tocopherols. These phytonutrients play various important roles as improving immune system, antioxidant activities, and health-promoting activities, thus are considered as important nutritional quality constituents of potato. Because of the presence of these important nutritional constituents in potato, potato is sometimes referred to as a wholesome food, though levels of these important nutrients are invariably quite low with reference to the RDA values for these nutrients. Therefore, there exists ample scope to further improve the nutritional value of potato and make it a truly wholesome food.

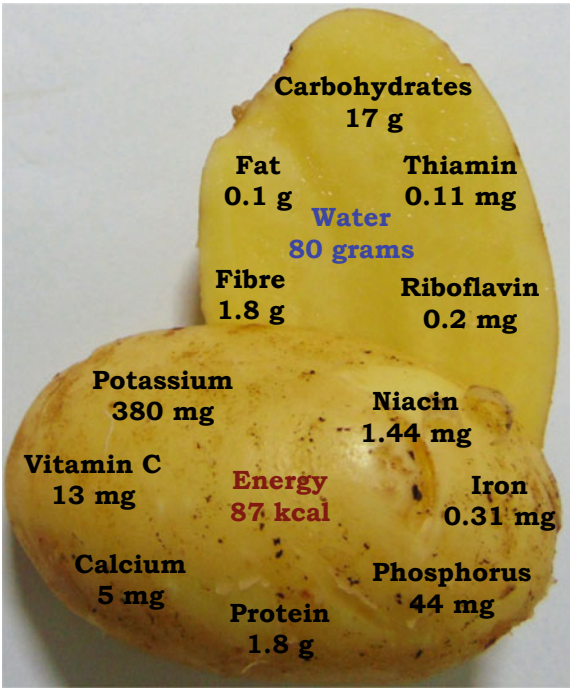

Fig. 15.1 Nutrient contents of potato (in 100 g after boiling in skin and peeling before consumption. Partially adapted from www.fao.org/potato-2008)

15.3 Biotechnological Tools for Assisting Conventional Plant Breeding

Recent advancements in the field of agricultural biotechnology have created a new domain to complement the methods of plant breeding. Genetic improvement can be achieved through conventional as well as nonconventional approaches. There are broadly three benefits to agriculture and crop improvement programs from use of biotechnology. These are: (i) reduction of the duration of the breeding programmes, (ii) to develop and multiply the healthy planting material, and (iii) genetically engineering the crop plants for trait improvement. The first two benefits hold true for all kinds of crop improvement and breeding programs. The third area, i.e., genetic engineering or recombinant DNA technology, is target trait specific.

From consumer point of view, the main quality traits in potato are nutrient content of the potato tubers, organoleptic characteristics including taste, flavor, and appearance. Therefore, for researchers improving the quality traits means improving any one, two, or all of these three characteristics of potatoes. Worldwide efforts are going on to develop the cultivars of potato with improved quality characteristics. Biotechnological tools are being continuously enriched and improved. Potatoes being one of the most important commodities for processing sector, its processing attributes are also crucial for determining their suitability for processing purposes (e.g., making

chips, French fries, and dehydrated products). This chapter is restricted to nutritional and processing trait improvement in potato using biotechnological approaches.

15.3.1 Genomic Resources for Biotechnological Applications in Potato

The generation of huge volume of the datasets of DNA sequences has gone much beyond everyone's imaginations. This has been mainly possible due to tremendous advancements in high throughputness of DNA sequencing technologies and the parallel development of sequencing storage servers and bioinformatics tools employed for DNA sequence assembly and annotations. Even in case of vegetable crops, the genome of the vegetables (including potato) belonging to more than 15 groups (tomato, potato, sweet potato, pepper, carrot, cabbage, turnip, radish, brinjal, cucumber, chenopodium, bitter gourd yam, beans, lettuce spinach, etc.) has been sequenced, and the genome sequence database is available in public domain (Table 15.2). This list of genome sequences of vegetable crops has expanded very rapidly. The assembly of these sequenced genomes is at different levels of assembly (chromosomes, scaffolds/contigs) (Table 15.2). Availability of genome sequences (including those of other vegetable crops) can be of great as a source of efficient gene isoforms for improving quality traits of potato through biotechnological approaches. Genome resequencing, single-nucleotide polymorphism (SNP) discovery through genotypic sequencing will be very useful in deciphering the genetic diversity at nucleotide sequence levels. This information in turn can be used for establishing the association between DNA/nucleotide variation and phenotypic/trait variability. The availability of genome sequences of various species within a clad/group may be very useful in performing genome-wide association mapping (GWAS) for various quality traits which will be vital for developing effective breeding strategies aiming at targeted quality trait(s) improvement. This may further help in identifying the more efficient alleles associated with desirable quality traits. However, this may need additional information about comparative kinetics of the enzymes encoded by these isoforms/alleles.

15.3.2 Transgenic-Based Tools for Quality Improvement of Potato

Genetic engineering has the application in introducing the specific traits into plants. It does not replace conventional breeding but add to the efficiency of crop improvement. It is possible due to the fact that plants are totipotent, enabling regeneration of a new plant from an isolated cell, tissue, or organ. Genetic engineering is the purposeful addition of a foreign gene or genes to the genome of an organism with the aim to

Table 15.2 Published sequenced genomes of vegetable crops

S. No.	Organism/Name	Common name	Bioproject	Size (Mb)	Scaffolds	Genes	Level of assembly
1.	*Solanum lycopersicum*	Tomato	PRJNA119	824	3224	30,336	Chromosome
2.	*Solanum pennellii*	Wild tomato	PRJEB5228	720	57,205	–	Contig
3.	*Solanum pennellii*	Wild tomato	PRJNA256426	926	12	32,519	Chromosome
4.	*Solanum arcanum*	Wild tomato	PRJEB5226	665	46,594	–	Contig
5.	*Solanum lycopersicum*	Tomato	PRJEB6302	760	13	–	Chromosome
6.	*Solanum lycopersicum, Heinz 1760*	Tomato	PRJNA41343	541	100,783	–	Scaffold
7.	*Solanum pimpinellifolium*	Wild tomato	PRJNA72351	688	309,180	–	Contig
8.	*Solanum melongena*	Egg plant	PRJDB1505	833	33,873	–	Scaffold
9.	*Capsicum annuum*	Pepper	PRJNA186921	2936	6478	41,504	Chromosome
10.	*Capsicum annuum*	Pepper	PRJNA223222	3064	35,797	35,845	Chromosome
11.	*Capsicum annuum var. glabriusculum*	Pepper	PRJNA193661	2768	16,998	–	Chromosome
12.	*Capsicum chinense*	Pepper	PRJNA331024	3071	87,978	34,974	Chromosome
13.	*Capsicum baccatum*	Hot pepper	PRJNA308879	3216	23,260	35,853	Chromosome
14.	*Solanum commersonii*	Wild potato	PRJNA269007	730	63,664	–	Scaffold
15.	*Solanum tuberosum*	Potato	PRJNA63145	706	14,854	33,410	Scaffold
16.	*Brassica oleracea var. oleracea*	Wild cabbage	PRJNA293438	489	32,886	53,670	Chromosome
17.	*Brassica oleracea var. capitata*	Cabbage	PRJNA174731	514	1816	–	Scaffold
18.	*Brassica juncea var. tumida*	Mustard	PRJNA285130	955	9746	–	Chromosome
19.	*Brassica napus*	Turnip	PRJEB5043	848	20,899	61,153	Scaffold
20.	*Spinacia oleracea*	Spinach	PRJNA396054	870	78,263	30,973	Scaffold

(continued)

Table 15.2 (continued)

S. No.	Organism/Name	Common name	Bioproject	Size (Mb)	Scaffolds	Genes	Level of assembly
21.	*Spinacia oleracea*	Spinach	PRJNA41497	494	103,502	21,539	Scaffold
22.	*Cucumis sativus*	Cucumber	PRJNA33619	196	190	20,396	Chromosome
23.	*Cucumis sativus*	Cucumber	PRJNA40333	324	13,113	–	Scaffold
24.	*Cucumis sativus*	Cucumber	PRJNA296786	343	8035	–	Contig
25.	*Chenopodium quinoa*	Chenopodium	PRJNA394242	1334	3487	58,734	Scaffold
26.	*Chenopodium suecicum*	Chenopodium	PRJNA326219	537	11,198	–	Scaffold
27.	*Chenopodium pallidicaule*	Chenopodium	PRJNA326220	337	3013	–	Scaffold
28.	*Raphanus sativus*	Radish	PRJNA344915	427	10,676	58,031	Scaffold
29.	*Raphanus sativus*	Radish	PRJNA259311	383	44,239	–	Chromosome
30.	*Raphanus sativus*	Radish	PRJDB1517	402	76,592	–	Scaffold
31.	*Raphanus sativus*	Radish	PRJDB707	383	40,123	–	Scaffold
32.	*Raphanus raphanistrum* subsp. *raphanistrum*	Wild radish	PRJNA209513	254	64,732	–	Contig
33.	*Dioscorea rotundata*	White yam	PRJDB3383	457	21	–	Chromosome
34.	*Manihot esculenta*	Cassava	PRJNA394209	582	2020	31,881	Chromosome
35.	*Manihot esculenta* subsp. *flabellifolia*	Cassava	PRJNA236442	391	54,016	–	Scaffold
36.	*Ipomoea batatas*	Sweet potato	PRJNA301667	837	28,461	–	Chromosome
37.	*Ipomoea trifida*	Wild sweet potato	PRJDB3230	513	77,400	–	Scaffold
38.	*Beta vulgaris* subsp. *vulgaris*	Sugarbeet	PRJNA41497	540	84,234	–	Scaffold
39.	*Daucus carota* subsp. *sativus*	Carrot	PRJNA326436	422	4826	36,299	Chromosome
40.	*Amaranthus hypochondriacus*	Grain amaranth	PRJNA214803	502	117,340	–	Scaffold

(continued)

Table 15.2 (continued)

S. No.	Organism/Name	Common name	Bioproject	Size (Mb)	Scaffolds	Genes	Level of assembly
41.	*Asparagus officinalis*	Garden *Asparagus*	PRJNA376608	1188	11,792	32,073	Chromosome
42.	*Vicia faba*	Faba bean	PRJEB8906	80	74,659	–	Contig
43.	*Momordica charantia, OHB3-1*	Bitter gourd	PRJDB4642	286	1052	21,623	Scaffold
44.	*Phaseolus vulgaris*	French bean	PRJNA221782	550	68,335	–	Chromosome
45.	*Lactuca sativa*	Lettuce	PRJNA68025	1134	876,110	–	Contig

Available in public domain database-NCBI; as on November 5, 2017

transfer the desired trait to the target plant. Genetic engineering physically removes the DNA from one organism and transfers the gene(s) for one or a few traits into another. Genetic engineering is mainly focused on the central dogma of biology. The components of central dogma, i.e., DNA, RNA, and proteins are manipulated to influence the targeted biological process, metabolic pathway or the trait. However, to do this we need quite a bit of information about the molecular, genetic, and biochemical basis of the target trait(s). Establishing the correlation between the gene (DNA) and the targeted trait is very crucial in achieving the success in genetic engineering. That is to identify the gene (s) which should/can be used for improving the quality trait in question. Once the genes have been identified, then the second question comes to search for the availability of the isoforms of the gene which are more efficient in improving the targeted trait. These information are very vital. Hence, the availability of genomic resources is proving to be very useful for genetic engineering.

All the five steps of plant genetic engineering (i) DNA extraction, (ii) gene cloning, (iii) designing suitable gene construct, (iv) plant transformation and regeneration, and (v) backcross breeding are common to all plants except the transformation and regeneration. Some plants are easy to transform and regenerate, whereas others are recalcitrant. The transformation methods usually employed are broadly classified into two categories, viz. direct transformation methods (electroporation or PEG-mediated transformation of protoplasts, biolistics, etc.) and indirect transformation methods (i.e., requiring an intermediate biological vector, usually the bacterium *Agrobacterium tumefaciens*). Successful transformation, however, relies on various phases, being the introduction and integration of DNA into the plant genome as well as the selection and regeneration of transformed cells. Plant regeneration is generally achieved via in vitro culture systems, using a range of explants and following two alternative pathways: de novo shoot organogenesis or somatic embryogenesis. As a result of worldwide R&D interventions, transformation and regeneration methods for potatoes are available now which otherwise initially were considered recalcitrant to in vitro regeneration. Due to these efforts, it is possible to genetically engineering these vegetables crops for desired traits.

Through transgenic-based approaches, the desired traits can be manipulated by two methods. These are overexpression of the specific gene(s), and repression or inhibition of the specific gene, or both these together. Various tools and constructs have been developed in order to perform these gene overexpression-mediated or gene repression (silencing)-mediated genetic engineering for improvement of the targeted trait. These aspects of the genetic engineering have been extensively described in various literatures. The employed tools/approaches are continuously being improved for their efficiency, precision, and biosafety. The transgenic technology has achieved great success in supplementing crop breeding.

15.3.3 Genome-Editing-Based Tools for Crop Improvement

Genome-editing biotechnological approach is the latest edition to the list of biotechnological approaches for crop improvement. Application of genome editing and their principal has been reviewed by many researchers (Xiong et al. 2015). Genome-editing technologies rely on engineered endonucleases (EENs) that cleave DNA in a sequence-specific manner due to the presence of a sequence-specific DNA-binding domain or RNA sequence. Through recognition of the specific DNA sequence, these nucleases can efficiently and precisely cleave the targeted genes. The double-strand breaks (DSBs) of DNA consequently result in cellular DNA repair mechanisms, including homology-directed repair (HDR) and error-prone nonhomologous end joining breaks (NHEJ), leading to gene modification at the target sites. There are various kinds of engineered endonucleases used for genome editing and can be very useful in improving quality traits of vegetable crops. These are very briefly described as follows.

15.3.3.1 Zinc Finger Nucleases System

Zinc finger nucleases (ZFNs) are the first-generation EENs that were developed following the discovery of the functional principles of the Cys2-His2 zinc finger (ZF) domains. Each ZF protein is able to recognize three contiguous nucleotide bases within the DNA substrate. A generic ZFN monomer is fused by two functional distinct domains: an artificially prepared Cys2-His2 ZF domain at the N-terminal and a nonspecific DNA cleavage domain of the Fok I DNA restriction enzyme at the C-terminal. The dimerization of the Fok I domain is crucial for its enzymatic activity. A ZFN dimer composed of two 3- or 4-ZF domains recognizes an 18- or 24-base target sequence that, statistically, forms a unique site in the genomes of most organisms. ZFNs have been successfully applied to gene modification in model plants (*Arabidopsis*, tobacco, maize, etc.). However, obtaining functional ZFNs requires an extensive and time-consuming screening process. Further, ZFNs have other limitations, such as off-target effector even toxic to the host cells. These shortcomings limit the application of ZFNs in plant genome editing.

15.3.3.2 Transcription Activator-Like Effector Nucleases System

A newly engineered endonuclease, i.e., transcription activator-like effector nucleases (TALENs), has rapidly emerged as an alternative to ZFNs for genome editing. The broad applications of TALENs were transcription activator-like (TAL) effectors that are secreted by the plant pathogenic bacteria *Xanthomonas*. After been pumped into host cells, the TAL effectors enter the nucleus and bind to effector-specific sequences in the host gene promoters and activate transcription. The DNA recognition property of the TAL effectors is mediated by tandem amino acid repeats (34 residues in length).

Two hypervariable amino acids known as repeat-variable di-residues (RVDs) located at the 12th and 13th positions in each repeat determine the binding specificity of the TAL effectors. The TALEN monomer is fused by two independent domains: a customizable DNA-binding domain at the N-terminal and a nonspecific Fok I nuclease domain at the C-terminal. Due to easier manipulation, the genes modified by TALENs have been successfully used in rice, wheat, *Arabidopsis*, potato, tomato, etc.

15.3.3.3 Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated 9 System

Recently, a new class of genome-editing technology, i.e., the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated) system, has been developed. CRISPRs were firstly identified in the *Escherichia coli* genome in 1987 as an unusual sequence element consisting of a series of 29-nucleotide repeats separated by unique 32-nucleotide "spacer" sequences. Later, repetitive sequences with a similar repeat–spacer–repeat pattern were identified in other bacterial and archaeal genomes, but the functions of these repeats remained obscure until 2005 when three independent research groups found the spacer sequence was identical to some part of the viral and plasmid sequence. Further investigations indicated that CRISPRs function through an RNA interference-like mechanism to recognize and cleave foreign DNA. The type II CRISPR/Cas from *Streptococcus pyogenes*, a short CRISPR RNA (crRNA), is able to recognize a complementary stretch of nucleotides in alien DNA and determines the sequence specificity. In addition, a transactivating crRNA (tracrRNA) is required to form a ribonucleoprotein complex with Cas9 nuclease to generate site-specific DSBs. Later, investigators found that the components of crRNA and tracrRNA could be combined into a single RNA chimera, which was termed as guide RNA (gRNA). Efficient cleavage also requires the presence of the protospacer adjacent motif (PAM) in the complementary strand following the recognition sequence. Various interventions have been carried out in CRISPR/Cas method to improve its target specificity. Presently, this technology is being applied in gene modification in various plants and holds great promise for nutritional quality improvement of potato as well.

15.4 Quality Traits of Potato Targeted Through Biotechnological Interventions

As discussed in previous sections, potato plays an important role in diet due to its nutritional content. In addition to have nutritional importance of potato as a staple food/vegetable, potatoes are also one of the most widely used food commodities for a wide range of processed products. Hence, their processing attributes may also be

considered as quality traits. Potatoes do contain some anti-nutritional factors, and thus reducing levels of these anti-nutritional factors also become improvement in quality. Improvement in these quality traits of potato employing biotechnological approaches will be described below.

15.4.1 Nutritional Quality Improvement

15.4.1.1 Phenolic Compounds

Phenolics are a diverse group of tens of thousands of different compounds. Many phenolics occur as derivatives formed by condensation or addition reactions. Chemically, a phenolic is a compound characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. Some phenolic compounds are effective against diseases or have other health-promoting qualities including effects on longevity, mental acuity, cardiovascular disease, and eye health (Scalbert et al. 2005). Phenolics are the most abundant antioxidants in the diet. Upon consumption, phenolics are metabolized by digestive and hepatic enzymes, by the intestinal microflora and have a wide range of bioavailability (Manach and Donovan 2004).

Potatoes are an important source of dietary phenolics. Phenolic compounds belonging to various classes are present in potato. These include: (i) phenolic acids (chlorogenic acid, caffeic acid, coumaric acid, protocatechuic acid, vanillic acid, ferulic acid, cryptochlorogenic acid, neochlorogenic acid, gallic acid, p-hydroxybenzoic acid, etc.), flavonols (rutin, kaempferol rutinose, quercetin-3-o-glu-rut), flavan-3-ols (catechin, epicatechin), anthocyanidins (delphinidin, cyanidin, pelargonidin, peonidin, malvidin, anthocyanins). Variations in these phenolic compounds in potato genotypes have been reported by several studies (Table 15.3) and reviewed by various researchers (Akyol et al. 2016). As some genotypes of potato have more phenolics than other vegetables (such as tomatoes, peas, onions, French beans, cucumbers, while cabbage, carrots, lettuce), potatoes can be a substantial source of phenolics in the diet and compare very favorably to other vegetables (Chun et al. 2005). Existence of variation of several folds in phenolic content in potatoes envisions the potential to further increase its nutritional value by more fully utilizing existing germplasm. For example, a study of 74 Andean potato landraces revealed an 11-fold variation in total phenolics and a high correlation between phenolics and total antioxidant capacity (Andre et al. 2007a). Similarly, Navarre et al. (2011) screened tubers for phenolics and found over a 15-fold difference in the amount of phenolics in different potato genotypes. Although majority of phenolic compounds are found in greater concentrations in the skin, but significant quantities are also present in the flesh Silva-Beltran et al. (2017), overall the flesh typically contains more phenolics than the skin on a per tuber basis because majority of the fresh weight of a mature potato is contributed by the flesh. These main phenolic compounds found in potato have been briefly described as follows.

Table 15.3 Concentrations of the main phenolic compounds in potato

Phenolics class	Phenolic compounds	Range (mg/100 g dry extract)	References
Phenolic acids	Chlorogenic acid	27.6	Kanatt et al. (2005)
		100.0–220.0	Shakya and Navarre (2006)
		17.4–1274.6	Andre et al. (2007a)
		47.0–283.0	Leo et al. (2008)
		17.3–1468.1	Mäder et al. (2009)
		21.0–40.0	Navarre et al. (2009)
		60.0–292.0	Navarre et al. (2010)
		0.2–2193.0	Deusser et al. (2012)
	Caffeic acid	0.1–0.2	Shakya and Navarre (2006)
		5.0–50.0	Leo et al. (2008)
		1.1–172.4	Mäder et al. (2009)
		2.0–6.9	Navarre et al. (2009)
		0–41.6	Deusser et al. (2012)
	Coumaric acid	0–9.2	Leo et al. (2008)
		0–1.6	Mäder et al. (2009)
	Protocatechuic acid	0–7.6	Mäder et al. (2009)
	Vanillic acid	0–22.4	Mäder et al. (2009)
	Ferulic acid	0.6–9.0	Leo et al. (2008)
		0–3.9	Mäder et al. (2009)
		0–1.4	Deusser et al. (2012)
	Cryptochlorogenic acid	16.0–27.0	
		3.1–163.3	
		8.0–59.0	
		0.1–168.3	
	Neochlorogenic acid	2.9–9.9	
		49.2–91.2	
		0.5–1.5	
		3.0–11.0	
		0.1–87.6	
	Gallic acid	0–1.0	Mäder et al. (2009)
	p-hydroxybenzoic acid	0–7.8	Mäder et al. (2009)
Flavonols	Rutin	0.5–2.6	Shakya and Navarre (2006)

(continued)

Table 15.3 (continued)

Phenolics class	Phenolic compounds	Range (mg/100 g dry extract)	References
		0.6–1.3	Navarre et al. (2010)
		0–12.2	Deusser et al. (2012)
	Kaempferol rutinose	0.5–1.7	Navarre et al. (2010)
	Quercetin-3-o-glu-rut	2.5	Shakya and Navarre (2006)
Flavan-3-ols	Catechin	43.0–204.0	Leo et al. (2008)
		0–1.5	Mäder et al. (2009)
		0–1.4	Deusser et al. (2012)
Anthocyanidins	Anthocyanins	1.4–163.3	Andre et al. (2007b)
		87.0	Han et al. (2007)
		953.8–1630.3	Andre et al. (2007b)
		21.0–109.0	Kita et al. (2013)

Adapted from Akyol et al. (2016)

Phenolic Acids

Phenolic acids and their derivatives are a diverse class of phenolic compounds made by plants. Phenolic acids are derivatives of benzoic and cinnamic acids. The most abundant benzoic acid derivatives are p-hydroxybenzoic, vanillic, syringic, and gallic acids, while common cinnamic acid derivatives include p-coumaric, caffeic, ferulic, and sinapic acids. The derivatives differ in the degree of hydroxylation and methoxylation of the aromatic ring. Phenolic acids are produced in plants via shikimic acid through the phenylpropanoid pathway. The phenolic acids reported to be present in potato tubers are briefly being discussed below.

Chlorogenic Acid

The most abundant phenolics in tubers are caffeoyl-esters. Of the caffeoyl-esters, chlorogenic acid (CGA) comprises over 90% of a tuber's total phenolics (Malmberg and Theander 1985). CGA acid is known to provide protection against degenerative, age-related diseases, may reduce the risk of some cancers and heart disease and have anti-hypersensitive anti-viral and anti-bacterial properties (Yamaguchi et al. 2008; Nogueira and do Lago 2007). The biosynthetic pathway of CGA in plants is depicted in Fig. 15.2. This CGA biosynthetic pathway can thus be engineered for increasing the CGA content in potato. Concerns have been shown about developing high phenolic potatoes that whether they would have unacceptable levels of browning or after cooking darkening. However, studies have shown that neither the amount of total phenolics, CGA nor polyphenols oxidase correlated with the amount of browning observed in fresh-cut potatoes and that they were not rate-limiting in the development

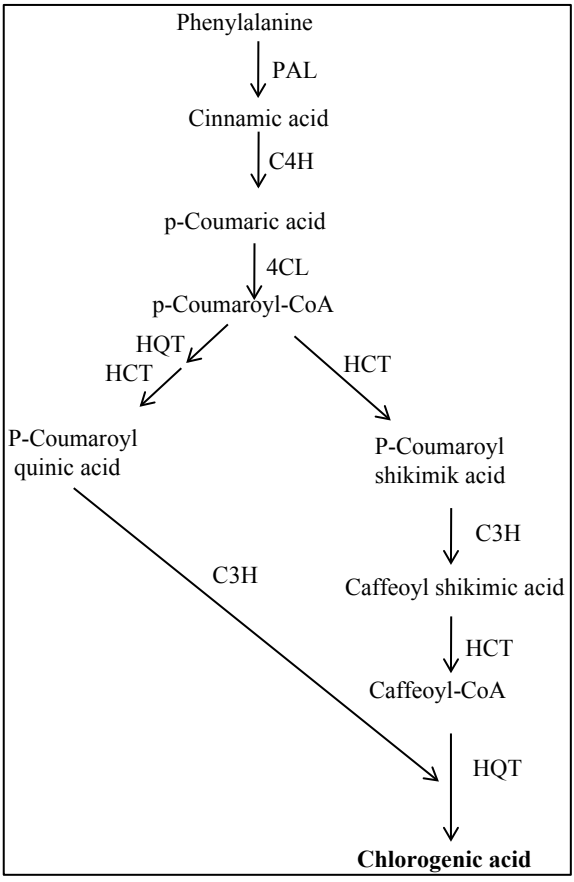

Fig. 15.2 Biosynthesis of chlorogenic acid in potato. PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumaroyl:CoA-ligase; HCT: hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3H: *p*-coumarate 3-hydroxylase; C4H: cinnamate 4-hydroxylase; HQT: hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase

of browning (Cantos et al. 2002). Further, using QTL approach, Werij et al. (2007) found no correlation between browning and CGA.

Flavons and Flavan-3-ols

Potatoes contain flavonols such as rutin, kaempferol rutinose, and quercetin-3-o-glu-rut, but have not been thought to be important source of dietary flavonols. Numerous studies have suggested flavons having multiple health-promoting effects, including reduced risk of heart disease, lowered risk of certain respiratory diseases, such as asthma, bronchitis, and emphysema, and reduced risk of some cancers including prostate and lung cancer. One group showed that flavonols increased in fresh-cut tubers, observing concentrations up to 14 mg/100 g FW and suggested that because of the large amount of potatoes consumed, they can be valuable dietary source (Tudela et al. 2002). Various studies have reported the presence of variations in the levels of these flavons in various potato genotypes (Table 15.3). Flavan-3-ols

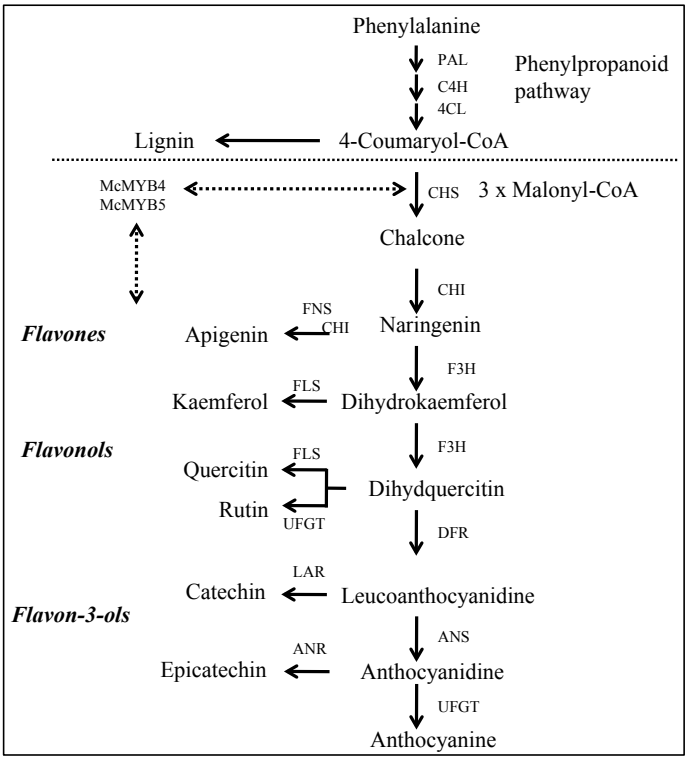

Fig. 15.3 Biosynthesis of flavons, flavan-3-ols and anthocyanins in potato. PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate-CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3 9 H: flavonoid 3 9–hydroxylase; DFR: dihydro-flavonol 4-reductase; FNS: flavone synthase; FLS: flavonol synthase; LAR: leucoanthocyanidin reductase; ANS: anthocyanidin synthase; UFGT: UDP glucose: flavonoid-3-O-glycosyltransferase

(sometimes referred to as flavanols) are derivatives of flavans and include catechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, proanthocyanidins, theaflavins, and thearubigins. Of these, some flavan-3-ols found in potato are enlisted in Table 15.3. Biosynthetic pathway of flavons and flavan-3-ols in plant is depicted as Fig. 15.3.

Anthocyanins

Potatoes, particularly colored-fleshed cultivars, can contain substantial amounts of anthocyanins, compounds that can function as antioxidants and have other health-promoting effects. Anthocyanins from potatoes have been found to have anti-cancer properties (Reddivari et al. 2007). A wide range of variations in anthocyanin content in potato have been reported (Table 15.3). Lewis et al. (1998) screened 26 colored-

fleshed cultivars for anthocyanin content and found up to 7 mg/g FW in the skin and 2 mg/g FW in the flesh. Oertel et al. (2017) screened 57 cultivars of potato for anthocyanin contents. Another study evaluated 31 colored genotypes and found a range of 0.5–3 mg/g FW in the skin and up to 1 mg/g FW in the flesh (Jansen and Flamme 2006). Brown et al. (2005) evaluated several genotypes for anthocyanins and found that whole tubers contained up to 4 mg/g FW and that anthocyanin concentration correlated with the antioxidant value. In 2005, Parr et al. reported the compounds called kukoamines in potatoes. These compounds are phenolic–polyamine conjugates and had previously only been found in a Chinese medicinal plant, in which they were being studied because they lower blood pressure. Roles of tuber polyamines include regulation of starch biosynthesis, calystegine synthesis, disease resistance, and sprouting (Tanemura and Yoshino 2006; Matsuda et al. 2005). Using high-throughput HPLC analysis, 30 putative polyamines were detected in potato tubers (Shakya and Navarre 2006).

Biosynthetic pathway of anthocyanins in plant is depicted as Fig. 15.3. It has been demonstrated that tuber-specific expression of the native and slightly modified MYB transcription factor gene StMtf1(M) activates the phenylpropanoid biosynthetic pathway. Compared with untransformed controls, transgenic tubers contained fourfold increased levels of caffeoylquinates, including CGA (1.80 mg/g dry weight), while also accumulating various flavonols and anthocyanins. Subsequent impairment of anthocyanin biosynthesis through silencing of the flavonoid-3′,5′-hydroxylase (F3′5′h) gene resulted in the accumulation of kaempferol-rut (KAR) to levels that were approximately 100-fold higher than in controls (0.12 mg/g dry weight). The biochemical changes were associated with increased expression of both the CGA biosynthetic hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (Hqt) gene and the upstream chorismate mutase (Cm) and prephenate dehydratase (pdh) genes. Field trials indicated that transgenic lines produced similar tuber yields to the original potato variety. Processed products of these lines retained most of their phenylpropanoids and were indistinguishable from untransformed controls in texture and taste (Rommens et al. 2008).

15.4.1.2 Carotenoids

Carotenoids are the second most abundant naturally occurring pigments on earth, with more than 750 members. Carotenoid pigments are mainly C40 lipophilic isoprenoids and synthesized in all photosynthetic organisms (bacteria, algae, and plants) and range from colorless to yellow, orange, and red, with different degree of variations. Carotenoids have numerous health-promoting properties. Some carotenoids are precursors of vitamin A and prevent human age-related macular degeneration, and some are potent antioxidant and are considered to prevent prostate cancer and cardiovascular disease. In humans, carotenoids also serve as antioxidants and reduce age-related macular degeneration of the eye, the leading cause of blindness in the elderly worldwide. An increasing interest in carotenoids as nutritional sources of

pro-vitamin A and health-promoting compounds has prompted a significant effort in metabolic engineering of carotenoid content and composition in food crops.

Potatoes also contain lipophilic compounds such as carotenoids, though in lesser amount (Table 15.4). The yellow/orange flesh color found in some potatoes is due to carotenoids. The carotenoids' content of tubers in most potato cultivars ranges between 0.2 and 36 μg/g FW (Iwanzik et al. 1983; Brown et al. 1993; Andre et al. 2007a). This variation in carotenoid concentrations has been suggested to be regulated mainly at the transcriptional levels (Morris et al. 2006). The most abundant potato carotenoids are composed mainly of the xanthophylls lutein, antheraxanthin, violaxanthin, and xanthophyll esters. Carotenoids are synthesized in plastids from isoprenoid pathway (Fig. 15.4) and are accumulated in most plant organs. Various genes of these pathways have been characterized in a range of organisms, and understanding of the regulation of the carotenoids pathway has led to devising strategies for manipulating this pathway. Numerous groups have attempted to increase potato carotenoids using transgenic strategies. The strategy commonly used in plants is to increase the biosynthetic capacity by altering the carotenogenic enzyme activities. Overexpressing a bacterial phytoene synthase in tuber of the cultivar *Desiree* increased carotenoids from 5.6 to 35 μg/g DW and changed the ratios of individual carotenoids. Beta-carotene concentrations increased from trace amounts to 11 μg/g DW and lutein levels increased 19-fold (Ducreux et al. 2005). Carotenoids have also been increased by the approaches that do not directly involve use of carotenoids biosynthesis genes, as shown by overexpression of the cauliflower *Or* gene in Desiree resulting in a sixfold increase in tuber carotenoids to about 20–25 μg/g DW (Lu et al. 2006). A twofold increase in carotenoids was observed in tubers overexpressing *Or* after six months of cold storage, but no such increase was observed in wild-type or empty-vector transformed plants (Lopez et al. 2008; Li et al. 2012). However, this is in contrast to earlier findings that potato cultivars undergo a decline in total carotenoids during cold storage (Morris et al. 2006). Cultivated diploid potatoes derived from *Solanum stenotomum* and *Solanum phureja* were found to contain up to 2000 μg/100 g FW zeaxanthin (Brown et al. 1993). A study of 24 Andean cultivars were found with almost 18 μg/g DW each of lutein and zeaxanthin and just over 2 μg/g DW of beta-carotene (Andre et al. 2007a). Overexpression of three bacterial genes in Desiree resulted in 20-fold increase in total carotenoids to 114 μg/g DW and a 360-fold increase in beta-carotene to 47 μg/g DW (Diretto et al. 2007). Bub et al. (2008) investigated whether zeaxanthin from genetically modified zeaxanthin-rich potatoes is bioavailable in humans and found that consumption of zeaxanthin-rich potatoes significantly increased chylomicron zeaxanthin concentrations suggesting that potentially such potatoes could be used as an important dietary source of zeaxanthin. Diretto et al. (2006) silenced the first dedicated step in the beta-epsilon branch of carotenoid biosynthesis, lycopene epsilon cyclase (LCY-e), by introducing, via *Agrobacterium*-mediated transformation, an antisense fragment of this gene under the control of the patatin promoter. Antisense tubers showed 2.5-fold increase in total carotenoids, with beta-carotene showing the maximum increase of up to 14-fold. The data suggested that epsilon cyclization of lycopene is a key regulatory step in potato tuber carotenogenesis.

Table 15.4 Carotenoids content reported in potato tubers (mg/kg DW* or FW**)

Carotenoid	Content	Potato cultivars	References
Total carotenoids			
	28.0*	Skin of tubers	Campbell et al. (2010)
	9.0*	Flesh of tubers	
	1.10–12.2*	Different cultivars	Hamouz et al. (2016)
	0.50–15.5*	Different cultivars	Fernandez-Orozco et al. (2013)
	0.58–1.75	Yellow cultivars	Breithaupt and Bamedi (2002)
	0.38–0.62	White cultivars	
	26.2*/5.69*	Yellow/red/purple	Brown (2005)
	8.0–20.0	Yellow–orange cvs.	
	26.0	Papa Amarilla cvs.	Brown et al. (2008)
	5.67	Inca-no-hitomi orange	Kobayashi et al. (2008)
	5.60–35.0*	Transgen. Desirée	Ducreux et al. (2005)
	3.0–36.0*	Andean landraces	Andre et al. (2007a)
	1.03–21.4	*S. phureja* accession	Bonierbale et al. (2009)
	2.57 ± 0.53*	Shetland Black	
	14.8 ± 2.22*	Red Laura	Burmeister et al. (2011)
	8.23 ± 2.98*	Boiled M. Twilight	
	1.51 ± 0.31*	Boiled Shetl. Black	Tierno et al. (2015)
	1.51 ± 0.31*	Boiled Shetl. Black	
Sum of carotenoid esters	0.41–1.31	Yellow and white	Breithaupt and Bamedi (2002)
Individual carotenoids			
All-*trans*-Lutein	1.12–17.7	Andean landraces	Andre et al. (2007b)
	0.55–1.89	*S. phureja* accession	Bonierbale et al. (2009)
	3.27–9.50*	Raw tubers	Clevidence et al. (2005)
	3.89–9.50*	Boiled tubers	
All-*trans*-Violaxanthin	trace–2.78	*S. phureja* accession	Bonierbale et al. (2009)
All-*trans*-Antheraxantin	0.03–3.54	*S. phureja* accession	Bonierbale et al. (2009)
All-*trans*-Zeaxanthin	18	Andean landraces	Andre et al. (2007b)
	12.9	*S. phureja*	Burgos et al. (2009)
	>10.0	*S. phureja*	Bonierbale et al. (2009)
	Trace–12.9	*S. phureja*	
	Trace–40*	Accession raw/boiled tubers	Clevidence et al. (2005)
All-*trans*-β-Carotene	2	Andean landraces	Andre et al. (2007b)

(continued)

Table 15.4 (continued)

Carotenoid	Content	Potato cultivars	References
	>0.1	*S. phureja* accession	Bonierbale et al. (2009)
Lutein-5,6-epoxide	Identified	Commercial, bred, old, and native cultivars	Fernandez-Orozco et al. (2013)
9-*cis*-Lutein	Identified		
13-*cis*-Lutein	Identified		
9-*cis*-Violaxanthin	+5,6-epoxide		
All-*trans*-Neoxanthin	+5,6-epoxide		
9′-*cis*-Neoxanthin	+5,6-epoxide		
Mutatoxanthin	Identified		
Luteoxanthin	+5,6 epoxide		
Neochrome	Identified		
All-*trans*-β-Cryptoxanthin	Identified		

Adapted from Lachman et al. (2016)

In 2006, Morris et al. engineered astaxanthin in potato tubers. Both *S. tuberosum* and *S. phureja* transgenic lines were produced that expressed an algal bkt1 gene, encoding a beta-ketolase, and accumulated ketocarotenoids. Two major ketocarotenoids were detected, ketolutein and astaxanthin. The level of unesterified astaxanthin reached 14 μg/g DW in some bkt1 expressing lines of *S. phureja* but was much lower in the *S. tuberosum*. Similarly, expression of *Erwinia uredovora* crtB gene encoding phytoene synthase in potato resulted in increased levels of carotenoids (Ducreux et al. 2005). The tuber of *S. tuberosum* L. cultivar Desiree normally produces tubers containing 5.6 μg carotenoid/g DW and tubers of *S. phureja* cultivar "*Mayan Gold*" contain carotenoid content of typically 20 μg carotenoid/g DW. In developing tubers of transgenic crtB Desiree lines, carotenoid levels reached 35 μg carotenoid/g DW and the balance of carotenoids changed radically compared with controls. Beta-carotene levels in the transgenic tubers reached 11 μg carotenoid/g DW, whereas control tubers contained negligible amounts and lutein accumulated to a level 19-fold higher than empty-vector transformed controls. The crtB gene was also transformed into *S. phureja* (cv. Mayan Gold), again resulting in an increase in total carotenoid content to 78 μg/g DW in the most affected transgenic line. In these tubers, the major carotenoids were violaxanthin, lutein, antheraxanthin, and beta-carotene. No increases in expression levels of the major carotenoid biosynthetic genes could be detected in the transgenic tubers, despite the large increase in carotenoid accumulation. Romer et al. (2002) genetically modified two potato varieties. By transforming with sense and antisense constructs encoding zeaxanthin epoxidase, zeaxanthin conversion to violaxanthin was inhibited. Both approaches (antisense and co-suppression) yielded potato tubers with higher levels of zeaxanthin. Depending on the transgenic lines and tuber development, zeaxanthin content was

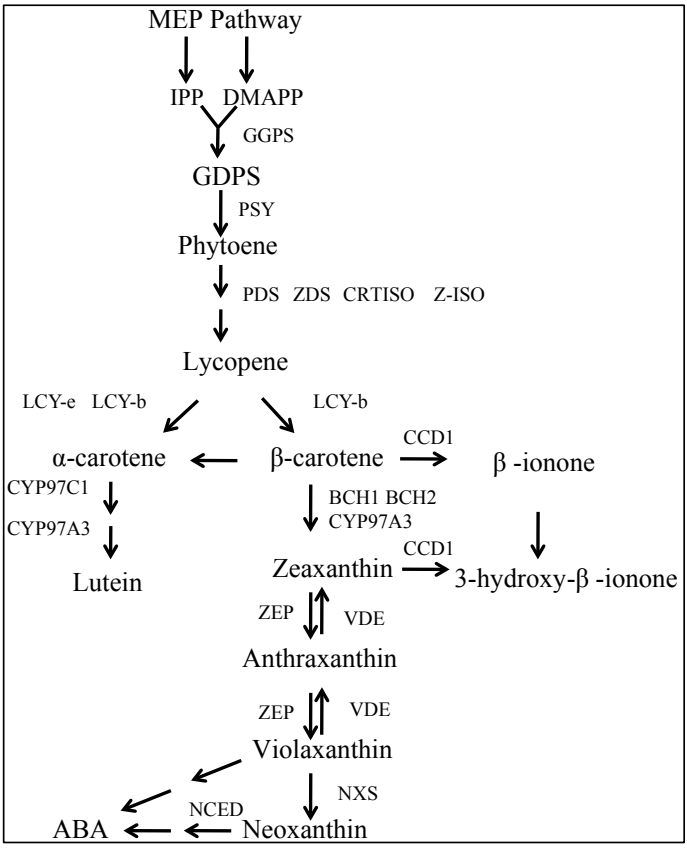

Fig. 15.4 Biosynthesis of carotenoids in potato. GGPS: geranylgeranyl pyrophosphate synthase; PSY: phytoene synthase; PDS: phytoene desaturase; ZDS: f-carotene desaturase; CRTISO: carotenoid isomerase; LCY-ε: lycopene ε-cyclase; LCY β: lycopene β-cyclase; CHY-ε: ε-ring hydroxylase; CHY-β: β-carotene hydroxylase; VDE: violaxanthin de-epoxidase; ZEP: zeaxanthin epoxidase; NXS: neoxanthin synthase; CCD: carotenoid cleavage dioxygenase; NCED: 9-cis-epoxycarotenoids dioxygenase

elevated 4–130-fold reaching values up to 40–78 μg/g DW. As a consequence of the genetic manipulation, the amount of violaxanthin was diminished dramatically and in some cases the monoepoxy intermediate antheraxanthin accumulated. In addition, most of the transformants with higher zeaxanthin levels also showed increased total carotenoid contents (up to 5.7-fold) and some of them exhibited reduced amounts of lutein. The increase in total carotenoids suggested that the genetic modification affects the regulation of the whole carotenoid biosynthetic pathway in potato tubers.

15.4.1.3 Vitamins

Vitamins are a class of organic compounds, absolutely required for the maintenance of healthy life processes. Role of vitamins in maintaining human health via regulating metabolism and supporting the biochemical process related to the energy released from food or other sources in living organisms is well established. Vitamins are also important in the synthesis of hormones, enzyme activity, red blood cells, genetic materials, and neurotransmitters (Jube and Borthakur 2006). Although vitamins are required in small amounts, their capability of sustenance and their ability to perform biochemical functions is remarkable. Based on the solubility, vitamins have been grouped into water-soluble vitamins and fat-soluble vitamins. Fat-soluble vitamins are A, D, E, and K, and the rest are water soluble. Most of the vitamins have been found to act as coenzymes; some act as growth regulators, and most of them as antioxidants. Well-known human vitamin-related disorders include blindness (Vitamin A), beriberi (Vitamin B1), pellagra (Vitamin B3), anemia (Vitamin B6), neural defects in infants (Vitamin B9), scurvy (Vitamin C), sterility-related diseases (Vitamin E), and Rickettsia (Vitamin D). In potato, predominant vitamin is vitamin C (Camire et al. 2009). Potato also contains several B vitamins (folic acid, niacin, pyridoxine, riboflavin, and thiamin), the composition of which is given in Table 15.5. Vitamin in potatoes can be increased through fortification in processed foods, conventional breeding, or through use of transgenic techniques, a process known as biofortification. The major vitamins present in potato and the research outcome to increase these vitamins level in potato are discussed below.

Vitamin C

Predominant vitamin in potatoes is vitamin C (also known as L-ascorbic acid), which ranges from 84 to 145 mg/100 g DW depending on cultivar and soil composition (Camire et al. 2009). A medium red-skinned potato (173 g) provides about 36% vitamin C of the RDA according to the USDA database (Navarre et al. 2009). Vitamin C is an important component in nutrition with the property of antioxidant, immunoprotection, cardiovascular function improvement, prevention of ailments associated with connective tissues, and help in iron metabolism. Vitamin C is a cofactor for numerous enzymes, functioning as an electron donor. The best-known symptom of vitamin C deficiency is scurvy, which in severe cases is typified by loss of teeth, liver spots, and bleeding. More than 90% of vitamin C in human diets is supplied by fruits and vegetables. It has been suggested that 100–200 mg vitamin C should be supplied by human diets, and this quantity is expected to be increasing because of increasing stress in modern life. Therefore, it is valuable to increase vitamin C content in edible products of plant. In India, the available supply of vitamin C is 43 mg/capita/day, and in the different states of India, it ranges from 27 to 66 mg/day which is far below the recommended dose of 400 mg/day by ICMR (National Institute of Nutrition 2011).

Plants may have multiple vitamin C biosynthetic pathways; with all of the enzymes of the L-galactose pathway have been characterized (Laing et al. 2007; Wolucka and

Table 15.5 Nutrient composition of potato (*Solanum tuberosum*), white, flesh, and skin, raw per 100 g

Nutrient		Units	Value per 100 g
Proximates	Water	g	81.6
	Energy	kJ	288
	Protein	g	1.68
	Total lipid (Fat)	g	0.1
	Ash	g	0.94
	Carbohydrate, by diff.	g	15.7
	Fiber, total dietary	g	2.4
	Sugar, total	g	1.15
	Sucrose	g	0.28
	Glucose (destrose)	g	0.53
	Fructose	g	0.34
	Lactose	g	0
	Maltose	g	0
	Galactose	g	0
	Starch	g	13.5
	Available carbohydrate[2]	g	14.65
Minerals	Calcium, Ca	mg	9
	Iron, Fe	mg	0.52
	Magnesium, Mg	mg	21
	Phosphorous, p	mg	62
	Potassium, K	mg	407
	Sodium, Na	mg	6
	Zinc, Zn	mg	0.29
	Copper, Cu	mg	0.116
	Manganese, Mn	mg	0.145
	Selenium, Se	mg	0.3
Vitamins	Vitamin C	mg	19.7
	Thiamin	mg	0.071
	Riboflavin	mg	0.034
	Niacin	mg	1.066
	Pantothenic acid	mg	0.281
	Vitamin B-6	mg	0.203
	Folate, total	mcg	18
	Folic acid	mcg	0
	Folate, food	mcg	18

(continued)

Table 15.5 (continued)

Nutrient		Units	Value per 100 g
	Folate DFE	Mcg_DFE	18
	Choline, total	mg	11
	Betaine	mg	0.2
	Vitamin B12	mcg	0
	Vitamin A IU	IU	8
	Vitamin A RAE	Mcg_RAE	0

USDA National Nutrient Database, No. 11365

Van Montagu 2007). One study examined tuber vitamin C content in 75 genotypes and found concentrations ranging from 11.5 to 29.8 mg/100 g FW (Love et al. 2004). This study also reported that some genotypes had more consistent concentrations of vitamin C than others across multiple years or when grown in different locations and suggests that the year may have a bigger effect than location. Dale et al. (2003) measured vitamin A in 33 cultivars grown in three locations around Europe and found vitamin C in a range of 13–30.8 mg/100 g FW. Extensive research work has been undertaken at molecular levels of vitamin C biosynthetic pathway in plants. An outline of plant vitamin C biosynthesis pathway is represented in Fig. 15.5. This knowledge has made it possible to manipulate vitamin C content in several crops (including potato) using various approaches including genetic engineering based. Transfer of L-gulone-γ-lactone oxidase gene from rat to potato resulted in 40% increase in vitamin C (Jain and Nessler 2000). Overexpression of gene encoding enzyme D-galacturonic acid reductase (catalyzes reduction of D-galacturonic acid or L-galactonic acid in the pathway for ascorbic acid biosynthesis via uronic acids) from strawberry in potato gave rise to twofold increase in tuber ascorbate content with respect to wild-type plants (Hemavathi et al. 2010; Vathi et al. 2009, 2011). Qin et al. (2011) transformed potato with its native cytosolic- and chloroplastic-targeted *DHAR* cDNAs, each under the control of the *CaMV 35S* promoter. Overexpression of cytosol-targeted *DHAR* led to increased ascorbate content in both tubers and leaves while overexpressing the chloroplastic enzyme also affected leaf ascorbate content. Bulley et al. (2012) reported an up to threefold increase in ascorbate through the overexpression of a single potato gene, GDP-L-galactose phosphorylase. In another report, the potato transgenic lines were developed by overexpressing DHAR gene, driven by the CaMV35S constitutive promoter and a tuber-specific patatin promoter. The AsA level in tubers of patatin: DHAR transgenic lines showed an enhanced level (up to 1.3-folds) as compared to that of control plants (Young et al. 2008). In another report, two independent transgenic potato lines were developed by overexpression of cytosolic DHAR (Cyt DHAR) gene and chloroplast DHAR (Chl DHAR) gene (Qin et al. 2011). The Cyt DHAR gene considerably augmented DHAR activities and AsA contents in potato tubers and leaves, because overexpression of Chl DHAR gene could only increase DHAR activities and AsA contents in leaves, not in tubers. These results indicated that AsA level of potato is enhanced by increasing recycling ascorbate via

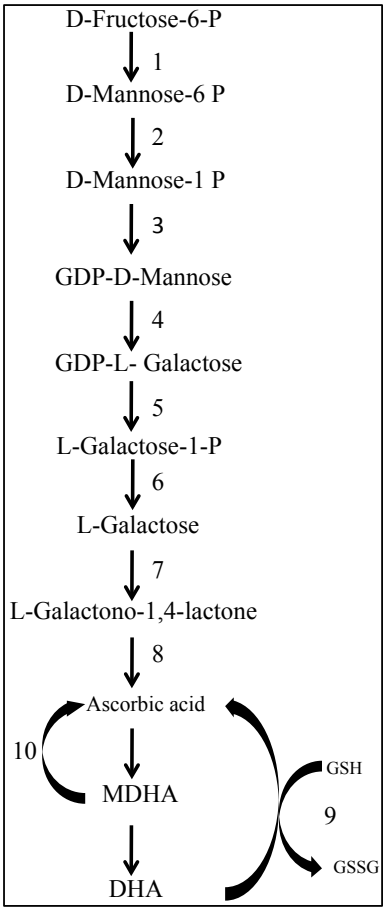

Fig. 15.5 Ascorbic acid biosynthesis and recycling pathways in plants. 1: mannose-6-phosphate isomerase; 2: phosphomannomutase; 3: GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase); 4: GDP-mannose-3′,5′-epimerase; 5: phosphodiesterase; 6: sugar phosphatase; 7: L-galactose dehydrogenase; 8: L-galactono-1,4-lactone dehydrogenase; 9: dehydroascorbate reductase; 10: mono-dehydro-ascorbate reductase

DHAR overexpression. Similarly, the potato transformation was done using the gene construct with potato isolate GGP (GDP-L-galactose phosphorylase) gene under the control of polyubiquitin promoter (tubers only). The molecular and biochemical study revealed that transgenic potato showed an increase in tuber ascorbate of up to threefold (Bulley et al. 2012).

Vitamin A

Vitamin A deficiency is one of the most prevalent nutrient deficiencies in many underdeveloped regions of the world, where it affects an approximately 250 million children under 5 years of age. Beta-carotene is the primary substrate for synthesis of vitamin A in humans. Plant pro-vitamin A carotenoids are the primary dietary precursors of vitamin A. While many fruits and vegetables have high levels of pro-

vitamin A carotenoids, staple crops contain low levels of these compounds, which contributes to the global prevalence of vitamin A deficiency. Vitamin A deficiency (VAD) is the leading cause of preventable blindness in children and increases the risk of disease and death from severe infections. To help combat vitamin A deficiency, a global effort is underway to increase pro-vitamin A content in major food crops including potato. Cultivated potato is extremely poor in pro-vitamin, i.e., β-carotene. However, metabolic engineering efforts to accumulate high levels of β-carotene in potato tubers proved successful. Ducreux et al. (2005) worked on two potato cultivars to increase the carotenoid content of potato tubers. *S. tuberosum* cv Desiree, which typically accumulates 5.6 μg/g DW carotenoids with negligible β-carotene content and *S. phureja* cv. Mayan Gold which typically accumulates 20 μg/g DW carotenoids. Both cultivars were transformed with the phytoene synthase gene (crtB) (for place of this enzyme in carotenoid biosynthetic pathway kindly see Fig. 15.4) from *E. uredovora*. Transgenic potato showed an accumulation of 35 total carotenoids and 11 μg/g DW β-carotene in developing tubers of Desiree and 78 μg/g DW in Mayan Gold tubers. In another study, the gene encoding lycopene ε-cyclase (Lcy-ε) (for place of this enzyme in carotenoid biosynthetic pathway kindly see Fig. 15.4) was targeted with a tuber-specific antisense construct in order to suppress epsilon cyclization of lycopene and direct the flux toward β-β-carotenoid branch (Diretto et al. 2006). Results showed a tuber-specific increase in the accumulation of β-carotene (up to 14-fold) and β-β-carotenoids (up to 25-fold) with a decrease in accumulation of lutein. When the β-carotene hydroxylation step of the β-β-carotenoid branch was targeted by tuber-specific antisense silencing of the beta-carotene hydroxylase (*chy1* and *chy2*) (for place of this enzyme in carotenoid biosynthetic pathway kindly see Fig. 15.4), a 38-fold increase in tuber β-carotene content was achieved (Diretto et al. 2007). Similarly, by silencing the β-carotene hydroxylase gene in potato using RNAi, Van Eck et al. (2010) were able to significantly increase beta-carotene content of tubers, even in lines that normally accumulate only low levels of zeaxanthin.

Vitamin E

Vitamin E (also known as tocopherols) is another essential nutrient for human health, but is consumed at suboptimal levels. The importance of vitamin E for reproductive health was recognized as early as 1922. Humans and other animals are not capable of synthesizing tocopherol (vitamin E) autonomously and must be obtained from their diet. The vitamin E (α-tocopherol) is only synthesized by photosynthetic organisms which show potent antioxidant activity and vital for human health, however, consumed at the suboptimal level. The metabolic pathways involved in tocopherol biosynthesis in plants have been deciphered to a greater extent. A generalized pathway of the vitamin E metabolic biosynthesis in plants is represented in (Fig. 15.6). In 2008, Crowell et al. reported the development of transgenic tuber over accumulating vitamin E where the transgenic potato lines developed via *Agrobacterium*-mediated transformation using two vitamin E biosynthetic genes, p-hydroxyphenylpyruvate dioxygenase (At-HPPD) and homogentisate phytyl transferase (At-HPT), isolated

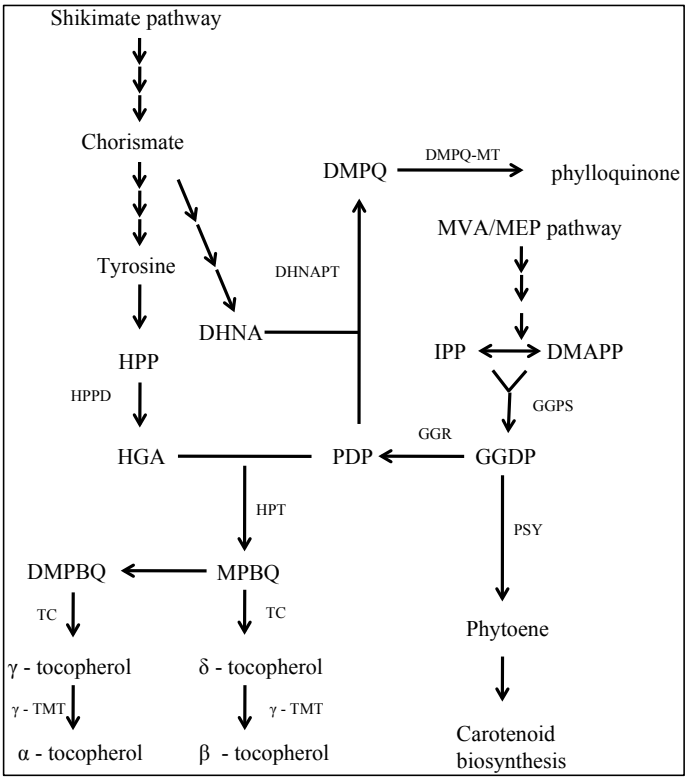

Fig. 15.6 Biosynthesis of tocopherols in plants. HPPD: p-hydroxyphenyl-pyruvate dioxygenase; HPT: homogentisate phytyltransferase; MPBQ: methylphytylbenzoquinone; MT: methyltransferase; γ-TMT: γ-tocopherol methyltransferase

from *Arabidopsis thaliana*. Biochemical and molecular analysis revealed that the overexpression of At-HPPD and At-HPT resulted in a maximum 266 and 106% increase in alpha-tocopherol, respectively, still lesser alpha-tocopherol than leaves or seeds. This might be limiting factors for tocopherol accumulation in potato tubers due to physiological and biochemical regulatory constraints. Overexpression of *Arabidopsis At-HPPD* and homogentisate phytyl transferase (*At-HPT*) genes in potato transgenics was carried in an attempt to increase vitamin E content of potato. *At-HPPD* resulted in maximum 266% increase in α-tocopherol, and overexpression of *At-HPT* yielded a 106% increase in potato.

Vitamin B9

Vitamin B_9, also known folates, is used as generic name to designate tetrahydrofolate (THF) and its on-carbon (C1) unit derivatives. Folates are essential micronutrients in the human diet. Folates are important cofactors involved in C1 unit transfer reac-

tions. Folates exist in various forms. All-native reduced folate derivatives are very sensitive to oxidative cleavage at the C9 and N10 bond; however, there are marked differences in stability of those species, 5-formyl-THF being the most stable natural folate, THF the least, and 5-methyl-THF intermediate. Folate deficiency is associated with the increase risk of neural tube defects, cardiovascular diseases, megaloblastic anemia, and some cancers (Bailey et al. 2003; Scott 1999). Unfortunately, folate intake is suboptimal in most of the world's populations, even in developed countries (Scott et al. 2000). Therefore, there is an urgent need to increase folate content and bioavailability in staple foods. Because of its large consumption worldwide, potato is an appealing target for enrichment.

Importance of folates in human diets urges to increase the folate levels in potato. Humans are not capable of synthesizing folates and thus require dietary supply. Plants represent the major source of folate in the diet. As such potato is in the lower range of folate contents among plant foods, even then potato is a well-known significant source of folates in the diet due to its high level of consumption more so that for its endogenous content. Several studies reported folate concentrations in potatoes of usually unspecified genotypes, and the reported values can vary substantially depending on the analytical method used. Values for folate concentrations in mature raw potato vary between 12 and 37 μg/100 g FW (Konings et al. 2001; Vahteristo et al. 1997) except a study by McKillop et al. (2002) who reported an exceptionally high folate concentration (125 μg/100 g FW). The USDA National nutrient Database for Standard Reference (SR20) gives values of 14 and 18 μg/100 g FW for raw potatoes. Goyer and Navarre (2007) determined total folate concentration of potato tubers from >70 cultivars, advanced breeding lines, and wild species and found showed an approximately threefold difference in folate values ranging from 0.46 to 1.37 μg/g DW or 11 to 35 μg/100 g FW. Vahteristo et al. (1997) determined that raw potatoes contain 21 μg/100 g FW of 5-methyl-THF, 3 μg/100 g FW of THF, and traces of 10-formyl folic acid, an oxidation product of 10-formyl-THF. Konings et al. (2001) showed that >95% of folates were present as a 5-methyl-THF derivative in potato tubers, the rest comprising 10-formyl folic acid and folic acid, and that total folate derivatives were >90% polyglutamylated. Therefore, polyglutamated forms of 5-methyl-THF seem to constitute most of the folate pool in potato tuber as is the case in most fruits and vegetables. This variation in folic acid content in various potato genotypes can be utilized through transgenic approaches for improving the folic acid content in popular commercial cultivars of potato. Nevertheless, improving folate contents using genetic engineering has been thought to be possible. As folate biosynthesis has been fairly delineated in recent years, metabolic engineering of the pathway is feasible. Recently, De Lepeleire et al. (2018) provided a proof of concept that additional introduction of HPPK/DHPS and/or FPGS, downstream genes in mitochondrial folate biosynthesis, enable augmentation of folates to satisfactory levels (12-fold) and observed folate stability upon long-term storage of tubers. This engineering strategy can serve as a model in the creation of folate-accumulating potato cultivars, readily applicable in potato-consuming populations suffering from folate deficiency.

Vitamin B6

Vitamin B_6 (chemically know as pyridoxine) is water soluble and like folate has several vitamins. Vitamin B_6 may be involved in more bodily functions than any other nutrient, is a cofactor for many enzymes, especially those involved in protein metabolism, and is also a cofactor for folate metabolism. Vitamin B_6 has anti-cancer activity, is a strong antioxidant, and is involved in hemoglobin biosynthesis, lipid and glucose metabolism, and immune and nervous system function (Tambasco-Studart et al. 2005; Theodoratou et al. 2008; Denslow et al. 2005). Possible consequences of deficiency include anemia, impaired immune function, depression, confusion, and dermatitis (Spinneker et al. 2007).

The most significant sources of Vitamin B_6 are animal proteins, starchy vegetables (potatoes), bananas, avocados, walnuts, peanuts, and legumes. Potatoes are an important source of dietary vitamin B_6 (Kant and Block 1990) with a medium-baked potato (173 g) providing about 26% of the RDA (USDA National Nutrient Database SR20). Very little research has been conducted on this vitamin in potato; thus, little is known about how much its concentration vary among genotypes. Rogan et al. (2000) had reported the content of Vitamin B_6 in potato in the range of 0.26–0.82 mg/200 g FW have been reported. Vitamin B_6 content varies substantially among the potato genotypes. There is thus great potential for improving potato further through increasing the content of this specific phytonutrient, by either breeding or genetic manipulation to fortify the B_6 vitamin as a healthy food resource for human nutrition. Work on elucidation of metabolism of vitamin B_6 in plants is in progress which may be of vital importance for improving the vitamin B_6 content in potatoes. Recently, Bagri et al. (2018) developed the of transgenic potato cv. Kufri Chipsona overexpressing key vitamin B_6 pathway gene, the PDXII from *A. thaliana* under the control of CaMV 35S constitutive promoter. Transgenic tubers exhibited 107–150% increase in vitamin B_6 accumulation in comparison to the untransformed controls potato.

15.4.2 Protein and Essential Amino Acids

Origin of name "Protein" (derived from the Greek word "*proteios*" means primary) itself justifies it as one of the primary components of the living cells and is the most important nutrient for humans. Lack of sufficient proteins in diet leads to deleterious effects on growth and development in human beings. The deleterious effects of diets that are sufficient in protein quantity but deficient in protein quality are well documented: poor growth, tissue wasting, and in severe cases, death. Lack of sufficient protein in diet is known as protein energy malnutrition (PEM), and this is the most lethal form of malnutrition and affects every fourth child worldwide. Building blocks of proteins are twenty common amino acids. Humans like other animals can only produce about half of the 20 common amino acids needed for life, the rest amino acids must be obtained via diet, and these amino acids are referred to as essential amino acids. Plant proteins contribute about 65% of the per capita supply of protein on

worldwide basis. Among plants, cereal grains, tubers, and food legumes are the most important suppliers of proteins. As the world's population increases (and with it the load on our agricultural resources), the need to make good-quality protein available efficiently and economically becomes increasingly important. The importance and urgency of providing humans with quality proteins are reflected in the growing scientific and industrial interest in augmenting the nutritive value of the world's protein sources. Major efforts have been made to enhance the overall protein content and/or to improve the essential amino acid composition of plant protein. The latter may be considered as improving the quality of the targeted protein(s). Most plant proteins are incomplete sources of amino acids. Among essential amino acids, methionine (Met), lysine (Lys), and tryptophan (Trp) are present in very low quantity as compared to other food sources (Table 15.6). This clearly shows that there is urgent need and ample scope for improving these essential amino acids in potato. Because of the importance of dietary protein and the fact that plants are its major source, development of strategies to increase protein levels and the concentration of essential amino acids in food crops is of primary importance in a crop improvement program. In potato, protein content ranges from 1 to 1.5% of tuber fresh weight (Ortiz-Medina and Donnelly 2003). Compared with other, it is negligible a source, potatoes are not typically considered to be good dietary protein sources due to their low overall protein content although it has excellent biological value of 90–100 (Camire et al. 2009). Keeping these facts in view, genetic engineering-based strategies and the efforts to enhance the protein quality/quantity and essential amino acids (specifically methionine, lysine and tryptophan) in various crop plants including potato have been targeted worldwide. Here, efforts been made in potato are described.

Table 15.6 Lys, Met, and Trp contents (mg/100 g food) in the major protein sources worldwide

Food	Lysine	Methionine	Tryptophan
Potatoes	*130*	*30*	*30*
Beans	1870	260	230
Peas	610	100	100
Soybean	1900	580	450
Maize	290	190	70
Barley	380	180	150
Rice	290	170	90
Wheat	380	220	150
Nuts	750	330	450
Pig meat	2200	750	310
Freshwater fish	2020	700	240
Marine fish, other	2050	600	240

Adapted from Le et al. (2016)

15.4.2.1 Role of Methionine, Lysine, and Tryptophan in Humans

Roles of these three essential amino acids, viz. lysine, methionine, and tryptophan have been described in several literatures. Methionine acts as a precursor for the synthesis of S-adenosylmethionine (SAM). SAM is a substrate involved in epigenetics and in fatty acid oxidation. Methionine also acts as an important methyl donor in human metabolism. Lack of methionine in diet leads to methylation-related disorders such as fatty liver, tumorigenesis, neurological disorders, and atherosclerosis. The limited availability of methionine leads to DNA strand breakage and fragmentation, which may be significant to the carcinogenic process (Garcia et al. 2011; Forges et al. 2007; Guthikonda and Haynes 2006; Scott and Weir 1998; Fowler 2005). Lysine plays several important roles in defense mechanism of humans. Lysine deficiency decreases defense ability of mammalian cells to viruses. Lysine deficiency is also the major cause of the osteoporosis in humans. Defects of lysine metabolism may result in familial hyperlysinemia due to genetic disorder (Gaby 2006; Sacksteder et al. 2000; Civitelli et al. 1992; Galvez et al. 2008). A diet deficient in methionine and lysine intake reduces biological value of plant-based nutrition to 50–70%, compared to a balanced diet with high abundance of essential amino acids. Unlike other amino acids, tryptophan acts as a precursor to several neurochemicals, such as serotonins and melatonin. Tryptophan deficiency in daily diet leads to several symptoms. Stresses caused by the loss of sleep were reportedly caused by the lack of tryptophan in daily food (Badawy 2013). Loss of lysine, methionine, and tryptophan in diet caused several symptoms include weight loss, decrease in muscle mass, and stress caused by losing sleep.

15.4.2.2 Genetic Engineering for Improving Methionine, Lysine, and Tryptophan in Potato

Genetic engineering exploits the metabolic pathway genes or the associated transcription factors. Hence, information at the genes levels of the metabolic pathways aimed for manipulation is prerequisite. As far as lysine, methionine, and tryptophan biosynthesis is concerned, their metabolic pathways in plants are fairly known. The success of the genetic approach has been mostly restricted to improving protein quality in model plants with enriched lysine, methionine, and tryptophan production. These are briefly described as follows. Lysine and methionine are synthesized by aspartate pathway within the chloroplast (Fig. 15.7). They share the initial three steps of this biosynthetic pathway. The first enzymatic step of the aspartate family is catalyzed by aspartate kinase which has multiple isoenzymic forms. Using ATP and Mg^{2+}, phosphorylation of aspartate leads to the formation of aspartyl phosphate which is subsequently oxidized to aspartate semialdehyde by aspartate semialdehyde dehydrogenase (ASDH). In the last step of the common pathway, ASD forms either dihydrodipicolinate (DHDP), a precursor of diaminopimelic acid and lysine, or O-phosphohomoserine (OPH). OPH may be channeled to threonine or methionine (Azevedo et al. 2006). Methionine is converted from OPH in three enzymatic steps:

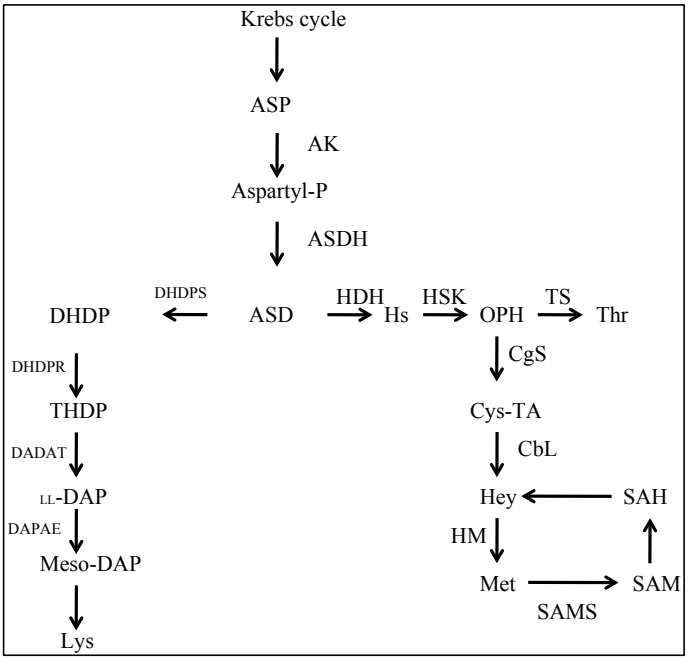

Fig. 15.7 Aspartate pathway leading to the biosynthesis of Met and Lys. Asp: Aspartate; AK: Aspartate kinase; ASD: aspartate semialdehyde; ASDH: aspartic semialdehyde dehydrogenase; DHDP: dihydrodipicolinate; DHDPS: dihydrodipicolinate synthase; HS: homoserine; HDH: homoserine dehydrogenase; OPH: O-phosphohomoserine; HSK: homoserine kinase; Thr: threonine; TS: Thr synthase; CysTA: cystathionine; CgS: cystathionine-synthase; HcY: homocysteine; CbL: cystathionine-lyase; SAH: Sadenosylhomocysteine; HM: homocysteine methyltransferase; SAM: S-adenosyl-methionine; SAMS: S-adenosyl-methionine synthetase; THDP: tetrahydrodipicolinate; DAPAE: DAP epimerase; DAP: diaminopimelate; DAPAT: DAP-aminotransferase

CgS catalyzes the formation of the thioether cystathionine from substrates of cysteine, the sulfur atom donor, and OPH by *trans*-sulfuration. The next step converts the intermediate to homocysteine and subsequently to methionine. In this mechanism, reactions are catalyzed by the enzymes CgS, cystathionine-lyase (CbL), and methionine synthase (MS), in that order. Almost 80% of methionine is converted into SAM, and the remainder takes part as a protein constituent (Hesse and Hoefgen 2003). Tryptophan biosynthesis too takes place in chloroplasts and is synthesized from chorismate (Fig. 15.8). Anthranilate synthase (AS) catalyzes the first reaction of the tryptophan biosynthesis which converts chorismate and an amine donor (usually glutamine) to form anthranilate; its activity is subject to feedback inhibition by Trp. In subsequent step, anthranilate phosphoribosylanthranilate transferase catalyzes a conversion of anthranilate and phosphoribosylpyrophosphate to phosphoribosylanthranilate and inorganic pyrophosphate. The third enzyme in the biosynthesis of tryptophan is phosphoribosylanthranilate isomerase (PAI) activity converting phosphoribosylanthranilate to L-(O-carboxyphenylamino)-L-deoxyribulose-5-phosphate

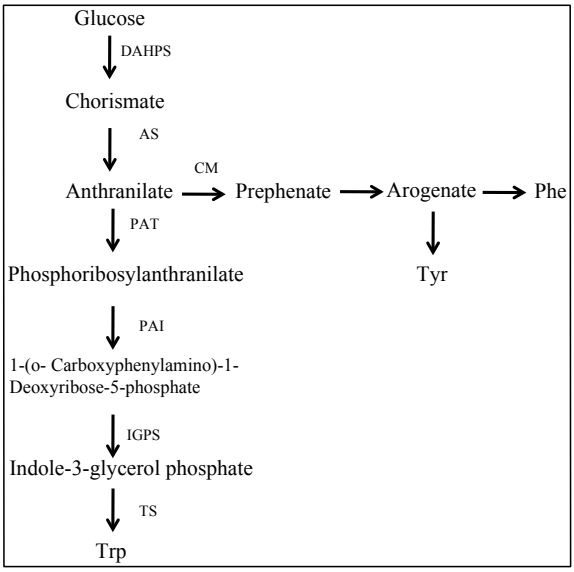

Fig. 15.8 Tryptophan biosynthesis pathway. AS: Anthranilate synthase; PAT: phosphoribosylanthranilate transferase; PAI: phosphoribosyl anthranilate isomerase; IGPS: indole-3-glycerol phosphate synthase; Trp: tryptophan; TS: Trp synthase; Tyr: tyrosine; Ser: serine; IAA: indole-3-acetic acid; Phe: phenylalanine; AH: arogenate dehydro; DAHPS: DAHP synthase

(CDRP). Then, indole-3-glycerol phosphate synthase (IGPS) accepts CDRP as the substrate which is transferred to indole-3-glycerol phosphate (Tzin and Galili 2010).

The major genetic engineering-based strategies for improving protein quantity/quality can be broadly grouped into three categories. These three groups are: (1) genetic engineering of essential amino acids. In potato tubers, in addition to amino acids present in proteins, some amino acids are "free" in the cytosol of seed cells and available to be digested within the tuber. These "free" amino acids also represent the pool available to the plant cell for protein synthesis and, to some extent, limit the amount and type of protein synthesized by the cell. Thus, genetic engineering to increase the level of amino acid synthesis has the potential to both remove some of the limitations to protein synthesis and enrich the "free amino acid content" of the plant. (2) Genetic engineering to enhance the levels of natural high-quality proteins within the plant tissue. In this approach, the gene copy number and transcription rate for specific genes are increased, or genes with appropriated essential amino acid profile from different organism can be imported for heterologous expression in the desired tissue (in case of potato, it is off-course tubers). (3) Improving the nutritional quality of protein plant synthesise, through protein engineering and/or design. Under this approach, the amino acid content of proteins expressed in potato tubers can be tailored, or entirely new, modified proteins with more desirable complement of amino acids can be designed and expressed. Although these three approaches can be followed separately, however, application of any one of these three strategies invariably results into more than one outcome in terms of improvement in protein quantity/protein quality/free amino acid levels. Therefore, various research works pertaining to improvement of proteins/amino acids in potato are discussed together under one section.

Efforts are being on for increasing content of various essential amino acids (methionine, lysine, tryptophan, threonine, etc.) in potato. Advances in biotechnology allowed the use of transgenic approach to increase the content of specific essential amino acids in a target plant. It was first demonstrated by the significant enhancement of methionine content in tobacco seed proteins through expressing transgene encoding a methionine-rich protein from Brazil nut (Altenbach et al. 1989). Beauregard et al. (1995) created an 11-kD synthetic protein, MBI, with 16% Met and 12% Lys, and transformed soybean using vectors targeted to seed protein storage bodies using appropriate leader sequences and seed-specific promoters. This was also achieved in a nonseed food crop, sweet potato (*Ipomoea batatas*), modified with an artificial storage protein gene (Egnin and Prakash 1997). These transgenic plants exhibited two- and fivefold increases in the total protein content in leaves and roots, respectively, over that of control plants. A significant increase in the level of essential amino acids, such as Met, Thr, Trp, Ile, and Lys, was also observed. In potato, higher methionine levels increase the nutritional quality and promote the typically pleasant aroma associated with baked and fried potatoes. Several attempts have been made to elevate tuber methionine levels by genetic engineering of methionine biosynthesis and catabolism. Chakraborty et al. (2000) developed transgenic potato overexpressing the sunflower albumin or an amaranth seed albumin (*AmA1*), driven under the constitutive promoters, which resulted in five- to sevenfold increase in total methionine level in tubers. Further analysis of transgenic potato lines with enhanced methionine amino acid via tuber-specific expression of a seed protein, *AmA1* (*Amaranth albumin* 1), revealed an increase in total protein contents up to 60% in comparison to the transformed potato (Chakraborty et al. 2010). Similarly, the methionine was also enhanced in transgenic potato by overexpression of gene encoding *PrLeg* polypeptide (isolated from *Perilla*), driven under the tuber-specific *patatin* promoter. This resulted in an increase in ~3.5-fold methionine in transgenic potato without changes in other amino acids or growth, development, and yield of the potato (Goo et al. 2013). It was also reported that higher isoleucine accumulation in transgenic tubers enhanced the methionine accumulation via methionine gamma-lyase (MGL) catabolism pathway (Huang et al. 2014). Recently, Kumar and Jander (2017) reported that overexpression of *A. thaliana* cystathionine γ-synthase gene in *S. tuberosum* increased methionine levels in tubers. Also, silencing *S. tuberosum* methionine γ-lyase gene, a gene encoding protein which causes degradation of methionine into 2-ketobutyrate, resulted in increase in methionine levels in tubers. Further, they reported that *S. tuberosum* cv. Désirée plants with *A. thaliana* cystathionine γ-synthase gene overexpression and *S. tuberosum* methionine γ-lyase gene silenced by RNA interference accumulated higher free methionine levels than either single-transgenic line. The paradise nut 2S seed protein is abundant Met residues (16 mol%). To explore the feasibility of further increasing Met content of this protein, modifications were made in the sequence region between the Cys-6 and Cys-7 codons of *PN2S* cDNA to contain 19, 21, and 23 mol% Met, respectively. All the three modified Met-rich *PN2S* were expressed, processed, and accumulated in transgenic tobacco seeds. The same modifications were also made in the Brazil nut 2S protein, and the chimeric genes were used to transform potato. Results revealed that the mutated Met-enriched BN2S proteins

were expressed and accumulated as well as normal 2S protein in the leaves and tubers of transgenic potato. The accumulation of the methionine-rich protein could make a significant enhancement in methionine levels in seed protein of transgenic potato (Tu et al. 1998). In another study, attempts were made to increase the Met content in potato tubers through heterologous overexpression of *Arabidopsis* cystathionine γ-synthase (*CgSΔ90*), which is not regulated by Met in potato plants and a storage Met-rich 15-kD zein in Desiree cultivar. There was sixfold increase in free Met content and in the Met content of the zein containing protein fraction of the transgenic tubers. In addition, in line with higher Met content, the amounts of soluble isoleucine and serine were also increased. However, all the lines with higher Met content *CgCΔ90* expressions were phenotypically abnormal showing severe growth retardation, changes in leaf architecture, and 40–60% reduction in tuber yield. Furthermore, the color of the transgenic tubers was altered due to reduced amounts of anthocyanin pigments.

In 1989, Yang et al. inserted the high essential amino acid encoding DNA (*HEAAEDNA*) into the chloramphenicol acetyltransferase (*CAT*) coding sequence to generate a CAT-HEAAE fusion protein. Transgenic study indicated that CAT-HEAAE protein was accumulated at 0.02–0.35% of total tuber protein in transgenic potato. Based on the structurally well-studied maize zeins, the group later designed and synthesized another artificial storage protein (ASP1) composed of 78.9% essential amino acids and estimated to possess a more stable storage protein like structure in plants (Kim et al. 1992). The 284-bp *asp1* gene, under the control of *CaMV 35S* promoter, was normally expressed in transgenic tobacco leaves resulting in the accumulation of relatively high levels of ASP1 proteins. Surprisingly, the overall levels of total amino acid and protein were found to be increased remarkably in transgenic potato. Gene silencing by RNAi technology also has been tried in potato to increase the essential amino acid content. The threonine synthase (*TS*) involved in synthesis of threonine in potato was targeted for silencing so as to divert the cycle and increase the Met content (Fig. 15.7). A reduction of 6% TS activity levels in transgenic potato which increased the methionine levels up to 30-fold developing on the transgenic line and environmental conditions and had no reduction in threonine (Zeh et al. 2001). Most enzymes in biosynthesis pathways leading to amino acids are inhibited by their end-products (allosteric regulation). 2S-sunflower seed protein has been characterized for its IgE-binding capacity; the protein possesses a significant amount of sulfur-containing amino acids (Hudson et al. 2005). This could be used to improve protein quality of other crops through genetic engineering. A chimeric gene encoding a methionine-rich Brazil nut (*Bertholletia excelsa*) protein contains over 18% methionine, whereas most proteins contain only a few percent of methionine. To increase methionine levels in plants, several transgenic approaches have been used. Cystathionine γ-synthase (CGS), the first committed enzyme in the methionine biosynthesis pathway, was overexpressed in transgenic potato plants. The transformation of PrLeg gene into potato, which contains low amounts of sulfur-containing amino acids, was found to enhance Met content in the tubers (Goo et al. 2013).

15.4.2.3 Minerals

Humans require various minerals to maintain health and for proper growth, and plants are essential source of such minerals (Welch 2002). Minerals can generally be classified as (a) major minerals [such as calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), cobalt (Co), manganese (Mn), nitrogen (N), and chlorine (Cl)] and (b) minor/trace minerals [such as iron (Fe), copper (Cu), selenium (Se), nickel (Ni), lead (Pb), sulfur (S), boron (B), iodine (I), silicon (Si), and bromine (Br)]. Importance of optimal intake of these minerals to maintain good health has been universally recognized (Avioli 1988). Potatoes are an important source of different dietary minerals. However, there are significant differences in major and trace mineral contents among different genotypes of potato (Randhawa et al. 1984; True et al. 1979). The minerals present in significant concentrations in potato are given in (Table 15.6). In addition to genetic factors, many other factors affect the mineral composition of potatoes; these include: location, stage of development, soil type, soil pH, soil organic matter, fertilization, irrigation, and weather. Therefore, the same genotype grown in different locations may have different mineral concentration due to these environmental factors (Burgos et al. 2007). The available information pertaining to the mineral content of potatoes and their improvement is described below.

In terms of mineral content, potato is best known as an important source of dietary potassium. Potassium plays a fundamental role in acid–base regulation, fluid balance, required for optimal functioning of the heart, kidneys, muscles, nerves, and digestive systems. Health benefits of sufficient potassium intake include reduced risk of hypokalemia, osteoporosis, high blood pressure, stroke, inflammatory bowl disease, kidney stones, and asthma. Potato is listed as providing 18% of the RDA of potassium. Potato qualifies for a health claim approved by the U.S. Food and Drug Administration, which states: "Diets containing foods that are good source of potassium and that are low in sodium may reduce the risk of high blood pressure and stroke." Potassium varies from 3550 to 8234 μg/gFW (Casanas et al. 2002; Rivero et al. 2003). The dietary reference intake of potassium for adult men and women is 3000–6000 mg per day. The US National academy of Sciences has recently increased the recommended intake for potassium from 3500 mg to at least 4700 mg per day. Besides potassium, phosphorus is the main mineral in potato tubers. It has many roles in the human body and is a key player for healthy cells, teeth, and bones. Inadequate phosphorus intake results in abnormally low serum phosphate levels, which affect loss of appetite, anemia, muscle weakness, bone pain, rickets/osteomalacia, susceptibility to infection, numbness and tingling of the extremities, and difficulty in walking. In potatoes, phosphorus ranges from ~1300 to 6000 μg/g DW (Lisinka and Leszczynski 1989). Daily requirement of phosphorus is 800–1000 mg. Potato is listed as providing 6% of the RDA of phosphorus. Calcium is important for bone and tooth structure, blood clotting, and nerve transmission. Deficiencies are associated with skeletal malformation and blood pressure abnormalities. The RDA for calcium is 600–1200 mg (Table 15.1). Potatoes are a significant source of calcium and have been shown to provide 2% of the RDA of calcium. Among 74 Andean landraces

screened, calcium ranged from 271 to 1093 μg/g DW (Andre et al. 2007b). Magnesium is required for normal functioning of muscles, heart, and immune system. Magnesium also helps maintain normal blood sugar levels and blood pressure. Potato magnesium levels range from 142 to 359 μg/g FW (Casanas et al. 2002; Rivero et al. 2003) and provides 6% of the RDA of magnesium. Manganese has a role in blood sugar regulation, metabolism, and thyroid hormone function. RDA for manganese is 2–10 mg. The range of potato manganese content has been reported from 0.73 to 3.62 μg/gFW (Rivero et al. 2003) to 9–13 μg/g DW (Orphanos 1980). Copper is needed for synthesis of hemoglobin, proper ion metabolism, and maintenance of blood vessels. The RDA for copper is 1.5–3.0 mg. Copper in potatoes varies from 0.23 to 11.9 mg/kg FW (Randhawa et al. 1984; Rivero et al. 2003). Like zinc, copper is also high in yellow-fleshed potatoes (Dugo et al. 2004).

Iron deficiency affects more than 1.7 billion people worldwide and has been called the most widespread health problem in the world by the World Health Organization. Due to severe iron deficiency, more than 60,000 women die in pregnancy and childbirth each year, and almost 500 million women of childbearing age suffer from anemia. Dietary iron requirements depend on numerous factors, for example, age, sex, and diet composition. Potato is a modest source of iron. Potato is listed as providing 6% of the RDA of iron. Iron content in cultivated potato tubers has been found in the range of 0.3–2.3 mg 100 g FW or 6–158 μg/g DW (True et al. 1979; Andre et al. 2007b). Potato iron has been suggested to be quite bioavailable because it has very low levels of phytic acid unlike the cereals. Zinc is needed for body's immune system to properly work and is involved in cell division, cell growth, and wound healing. Iron and zinc deficiencies result in decreased immune function and can interfere with growth and development (Zimmermann and Hurrell 2002). The RDA for zinc is 15–20 mg, and potato is listed as providing 2% of the RDA of zinc. The zinc content ranges from 1.8 to 10.2 μg/g FW (Andre et al. 2007b; Randhawa et al. 1984; Rivero et al. 2003). Yellow-fleshed potatoes from different cultivars contain zinc in 0.5–4.6 μg/g FW (Dugo et al. 2004).

Improving Mineral Content in Potatoes Using Genetic Engineering

Very few reports are there about research attempts improving mineral content in potato through biofortification. Because plants cannot synthesize these minerals, they must be acquired from soil. As a result, engineering of plant mineral content is quite different from modifications of improving other nutrition associated constituents such as proteins and vitamins that the plant itself synthesizes. There are four main strategies which can be employed for improving the mineral contents in potatoes (Fig. 15.9); these are: (i) improving minerals uptake from soil, (ii) increasing transport to storage organ, (iii) increasing storage capacity of sink, and (iv) decreasing anti-nutrient (phytic acid, phytase, etc.) components which reduce availability of the minerals. Research to improve the mineral composition of crop plants has mostly focused on iron content. Several reports exist in this particular area, most of which describe research that was performed on iron biofortification in rice crop. However,

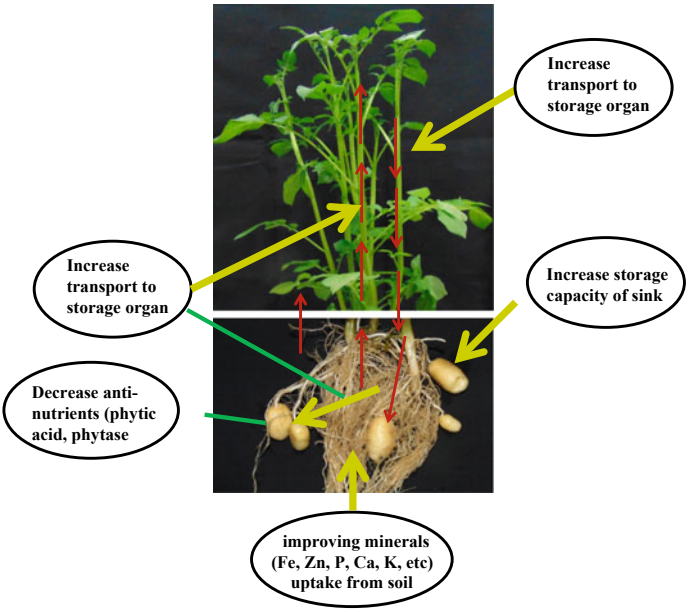

Fig. 15.9 Approaches for improving mineral content in potato. The four main strategies include: (i) improving minerals uptake from soil, (ii) increasing transport to storage organ, (iii) increasing storage capacity of sink, (iv) decreasing anti-nutrient (phytic acid, phytase, etc.) components which reduce availability of the minerals

very less research in this regard in potato has been carried out worldwide. An attempt was made to over express *Arabidopsis sCAX* (Cationic Exchanger 1) and H^+/Ca^{2+} transporter genes in potato. Transgenic tubers expressing *sCAX1* displayed up to threefold more calcium content compared to wild type without significant alteration in growth and development. The trait was also found to be stably inherited when monitored over three generations (Park et al. 2005). In other work, a chimeric, N-terminus-truncated *Arabidopsis* cation transporter (*CAX2B*) that contains a domain from *CAX1* for increased substrate specificity was over expressed in potato to improve calcium accumulation. The transgenic plants had 50–65% improved tuber calcium content relative to wild type, with stable inheritance and no deleterious effects on plant growth or development (Kim et al. 2006).

15.4.3 Reducing Anti-nutritional Factors

15.4.3.1 Glycoalkaloids

Steroidal alkaloids (SAs) and their glycosylated forms, i.e., steroidal glycosylated alkaloids (SGAs) are toxic compounds mainly produced by members of the Solanaceae and Liliaceae plant families. In humans and animals, steroidal alkaloids

are considered anti-nutritional factors because they affect the digestion and absorption of nutrients from food and might even cause poisoning. In spite of the first report on steroidal alkaloids nearly 200 years ago, much of the molecular basis of their biosynthesis and regulation remains unknown. It has been perceived that elaborating the knowledge regarding the steroidal alkaloids biosynthetic pathway, the subcellular transport of these molecules, as well as the identification of regulatory and signaling factors associated with steroidal alkaloids metabolism, will also provide the means to develop, through classical breeding or genetic engineering, crops with modified levels of anti-nutritional SAs (Cardenas et al. 2015). Recently, co-expression analysis and metabolic profiling revealed metabolic gene clusters in tomato and potato that contain core genes required for production of the prominent SGAs.

The presence of SGAs in potatoes has been of a particular concern due to their toxicity to humans (Friedman et al. 1997). In potatoes, SGAs are found in every plant organs (roots, tubers, stolons, stems, foliage, flowers, and fruits) with fresh weight concentrations ranging from 10 mg per kg (fresh weight) in tubers to 5000 mg per kg (fresh weight) in the flowers (Smith et al. 1996). Solanine and chaconine, derived from the aglycone solanidine, are the most prevalent glycoalkaloids found in cultivated potato (Dale et al. 1993). Solanine and solasonine have a common sugar moiety (solatriose) while chaconine and solamargine have chacotriose in common. The alkamine steroidal skeletons (aglycones) of the glycoalkaloids are classified into two groups, the spirosolanes and solanidanes, of which solasodine and solanidine are representatives, respectively. These compounds are derived from mevalonic acid. The use of wild germplasm in potato breeding is extensive and the main source of transmission of unusual SGAs (Väänänen et al. 2006).

Elimination of solanidine glycosylation has been found to decrease toxicity of edible tuber. Antisense DNA constructs of *SGT1* coding for solanidine galactosyl transferase involved in α-solanine biosynthesis, *SGT2* coding for solanidine glucosyltransferase involved in α-chaconine biosynthesis, or *SGT3* coding for sterol rhamnosyltransferase, the last step in the triose formation of α-chaconine and α-solanine (McCue et al. 2005, 2006, 2007), reduced the corresponding glycoalkaloids in transgenic potato plants. Antisense silencing of a potato gene encoding a sterol alkaloid glycosyl transferase (*sgt1*) resulted in complete inhibition of α-solanine accumulation. But this decrease was compensated by elevated levels of α-chaconine and resulted in wild-type total steroidal glycoalkaloids (SGA) levels in transgenic lines (McCue et al. 2005). Arnqvist et al. (2003) overexpressed soybean (*Glycine max*) type 1 sterol methyl transferase (*GmSTM1*) in potato (cv. Desiree) in an attempt to reduce glycoalkaloids. The transgenic potato showed decreased glycoalkaloid levels in leaves and tubers, down to 41 and 63% of wild-type levels, respectively. In 2002, Esposito et al. estimated the glycoalkaloid content in potatoes improved with nonconventional breeding approaches. They performed chemical analyses on two distinct groups of new potato genotypes. The first group contained clones transformed with the gene ech42 encoding for an endochitinase. The second included interspecific hybrids between the cultivated potato *S. tuberosum* and the wild species *Solanum commersonii*, obtained either by somatic fusion or by sexual hybridization. The results suggested that chitinase gene insertion did not alter other metabolic

pathways of potato tubers and did not cause unintentional pleiotropic effects. In interspecific hybrids, wide variability for all of the parameters analyzed was found. In a number of genotypes, glycoalkaloid levels were close to or lower than those of the control varieties, suggesting that selection for low glycoalkaloid content is possible. The results also indicated that glycoalkaloids from *S. commersonii* may be lost rapidly. Recently, co-expression analysis and metabolic profiling revealed metabolic gene clusters in tomato and potato that contain core genes required for production of the prominent SGAs (Cardenas et al. 2015).

15.4.3.2 Acrylamide and Allergens

Acrylamide has been classified as probable carcinogen in humans and has neurological and reproductive effects. It is formed from free asparagine and reducing sugars during high-temperature cooking and processing of common foods. Potato and cereal products are major contributors to dietary exposure to acrylamide. One of the promising approaches to reduce the acrylamide formation in plant-based processed products is to develop crop varieties with lower concentrations of free asparagine and/or reducing sugars, and of best agronomic practice to ensure that concentrations are kept as low as possible (Halford et al. 2012). Chawla et al. (2012) reported that simultaneous silencing of asparagine synthetase (Ast)-1 and -2 reduced asparagine formation and, consequently, reduces the acrylamide-forming potential of tubers. However, phenotypic analysis revealed that the phenotype of silenced lines appears normal in the greenhouse, but field-grown tubers were small and cracked. Assessing the effects of silencing StAst1 and StAst2 individually, they found that yield drag was mainly linked to down-regulation of StAst2. Interestingly, tubers from untransformed scions grafted onto intragenic StAst1/2-silenced rootstock contained almost the same low ASN levels as those in the original silenced lines, indicating that ASN is mainly formed in tubers rather than being transported from leaves. Further, field studies demonstrated that the reduced acrylamide-forming potential achieved by tuber-specific StAst1 silencing did not affect the yield or quality of field-harvested tubers.

Allergies to potatoes appear to be relatively uncommon. Patatin, the primary storage protein in potato unfortunately, has also been suggested to be major allergen in potato. Patatin may be cross-reactive for persons with allergy to latex, and children with atopic dermatitis appear to have increased sensitivity to this potato protein (Schmidt et al. 2002). However, boiling of potatoes reduce or nullify the allergic reaction. No significant work has been carried out to remove or minimize the allergic potential of potatoes. The biotechnological way to overcome this patatin-associated allergy may be developing potato cultivar engineered for the patatin protein at specific site(s) which is/are responsible for inducing the allergic response upon consumption.

15.4.4 Improving Carbohydrates Quality

After cellulose, starch is the second most abundant compound produced in higher plants. Starch represents the most important carbohydrate used for food and feed purposes. While cellulose is a structural component of plants, starch mainly serves as a compound to temporarily store energy that can be accessed at a later time point. Chemically, starch is an alpha-glucan (α-glucan) and composed of two types of polysaccharides: amylose and amylopectin (Fig. 15.10). Amylopectin is highly branched, leaving more surface area available for digestion. It is broken down quickly and thus produces a larger rise in blood glucose. On the other hand, amylose is a straight chain, which limits the amount of surface area exposed for digestion. Therefore, digestion of amylose is slow than that of amylopectin and hence is responsible for resistant nature of starch. Thus, improving the resistant starch content refers to increasing the amylose content of the target crop. Resistant starch provides health benefits such as glycemic control, control of fasting plasma triglyceride and cholesterol levels and absorption of minerals. In view of the industrial application and the nutritional benefits of resistant starch, researchers around the globe have been working to increase the RS content of the plants. The approaches for increasing the RS content in plants include natural selection, conventional breading as well as transgenic. All these approaches are based on biosynthetic pathways of starch metabolism. The key enzymes for starch biosynthesis are AGPase, starch synthases, and branching enzymes. Generation of the sugar nucleotide ADP glucose is catalyzed by AGPase. Starch synthases catalyze the polymerization of glucose residues resulting in formation of α-1,4 glucans. Branching enzymes cleave α-1,4 glucans and reattach the cleaved chain to an α-1,4 glucan chain by an α-1,6 glycosidic linkage, thereby forming a branch (Fig. 15.10).

As potato contains high starch, they have been genetically modified for increasing the resistant starch content (i.e., amylose content). In 2000, Schwall et al. developed very-high-amylose potato starch by manipulating starch branching enzymes through genetic engineering. They simultaneously inhibited two isoforms of starch branching enzyme to below 1% of the wild-type activities which resulted in altered starch granule morphology and composition. In these, potato amylopectin was found to be absent, whereas the amylose content increased to levels comparable to the highest commercially available maize starches. Expression of amylosucrase in potato resulted in larger starch granules with rough surfaces and novel physicochemical properties, including improved freeze–thaw stability, higher end viscosity, and better enzymatic digestibility. In 2005, Blennow et al. reported genetic engineering of potato tuber starch by simultaneous antisense suppression of the starch branching enzyme (SBE) I and II isoforms. Starch prepared from 12 independent lines, and three control lines were characterized with respect to structural and physical properties. The lengths of the amylopectin unit chains and the concentrations of amylose and monoesterified phosphate were significantly increased in the transgenically engineered starches. With the aim of increasing starch content, Regierer et al. (2002) modulated the adenylate pool by changing the activity of the plastidial adenylate

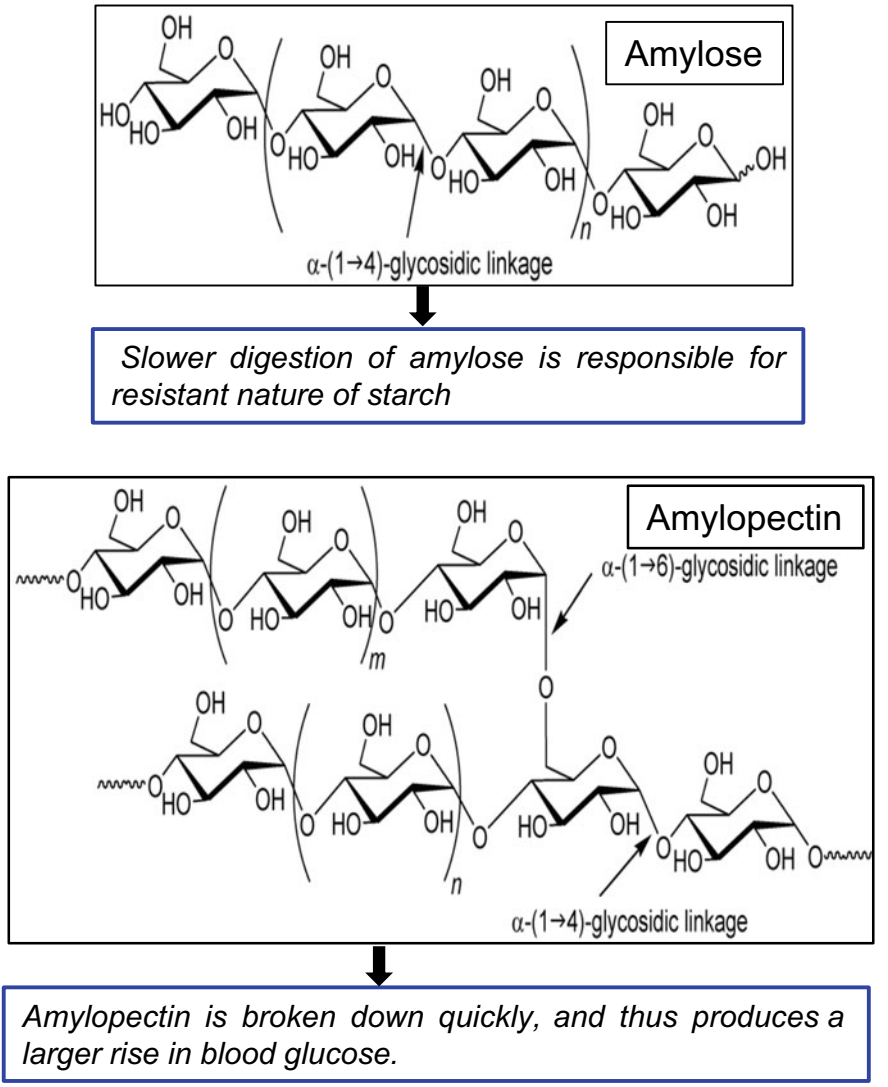

Fig. 15.10 Starch components in potato. Starch has two components, viz. amylose and amylopectin. Amylose is considered as resistant starch

kinase in transgenic potato plants. A substantial increase in the level of adenylates and, most importantly, an increase in the level of starch to 60% above that found in wild-type plants were observed.

Beaujean et al. (2000) demonstrated that it is possible to replace starch degradation using microbial enzymes via a system where the enzymes are produced directly in the plants, but active only at high temperature, thus offering novel and viable strategies for starch-processing industries. They reported for the first time that starch was degraded and glucose and fructose were produced directly when crushed potato tubers expressing a starch degrading bifunctional gene were heated for 45 min at 65 °C. To achieve this, we have constructed a fusion gene encoding the thermostable enzymes: alpha-amylase (*Bacillus stearothermophilus*) and glucose isomerase (*Thermus thermophilus*) under the control of the granule-bound starch synthase promoter. This enzymatic complex produced in transgenic tubers was only active at high temperature (65 °C). More than 100 independent transgenic potato plants were regenerated. The biochemical analyses performed on young and old tubers after high-temperature treatment (65 °C) revealed an increase in the formation rate of fructose and glucose by a factor of 16.4 and 5.7, respectively, in the transgenic tubers as compared to untransformed control tubers. Potato tuber pectin is rich in galactan (oligomer of beta-1,4-linked galactosyl residues). Oxenboll Sorensen et al. (2000) expressed a fungal endo-galactanase cDNA in potato under control of the granule-bound starch synthase promoter to obtain expression of the enzyme in tubers during growth. Analyses of transgenics revealed alterations in pectin composition. Monosaccharide composition of total cell walls and isolated rhamnogalacturonan I fragments showed a reduction in galactosyl content to 30% in the transformants compared with the wild type.

15.4.5 Improving Processing Attributes

Accumulation of reducing sugars (primarily glucose and fructose) in cold-stored potato tubers is referred to as "cold-induced sweetening" (CIS). CIS makes the cold-stored potatoes unfit for processing purposes such as chips and French fries making (Fig. 15.11). Two separate metabolic events are critical in determining a potato tuber's ability to produce sugars in the cold storage: the ability to form sucrose and the ability to hydrolyze sucrose to the reducing sugars glucose and fructose. The control of sucrose synthesis is controlled by several related enzymes while reducing sugar formation is more specifically related to level of vacuolar acid invertase activity. Role of vacuolar acid invertase in cold-induced sweetening has been demonstrated by various researchers (Fig. 15.12). Bhaskar et al. (2010) demonstrated that silencing the potato vacuolar acid invertase gene (VInv) prevented reducing sugar accumulation in cold-stored tubers. Potato chips processed from VInv silencing lines were light in color even when tubers were stored at 4 °C. Comparable, low levels of VInv gene expression were observed in cold-stored tubers from wild potato germplasm stocks that are resistant to cold-induced sweetening. Wiberley-Bradford et al. (2014) have

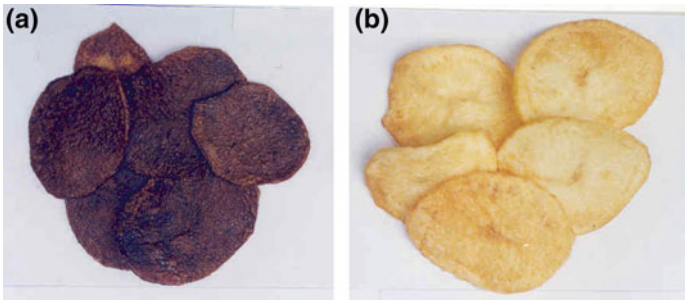

Fig. 15.11 Chips prepared from potatoes having high glucose content due to cold-induced sweetening (**a**); and from potatoes having low glucose content (**b**)

shown that vacuolar invertase (VInv) silencing significantly reduced cold-induced sweetening in stored potato tubers, likely by means of differential VInv expression early in storage. Zhu et al. (2014) suppressed the expression of Vacuolar Invertase gene (VInv) in cultivars Russet Burbank and Ranger Russet using RNA interference to determine if this approach could control sugar-end defect formation. Acid invertase activity and reducing sugar content decreased at both ends of tubers. Clasen et al. (2016) used transcription activator-like effector nucleases (TALENs) to knockout vacuolar invertase gene (VInv, which encodes a protein that breaks down sucrose to glucose and fructose) within the commercial potato variety, Ranger Russet. They isolated 18 plants containing mutations in at least one VInv allele, and five of these plants had mutations in all VInv alleles. Tubers from full VInv-knockout plants had undetectable levels of reducing sugars, and processed chips contained reduced levels of acrylamide and were lightly colored. These results provide a framework for using TALENs to quickly improve traits in commercially relevant autotetraploid potato lines.

Rommens et al. (2006) improved potato storage and processing characteristics through all-native DNA transformation. They simultaneously lowered the expression of Ranger Russet's tuber-expressed polyphenol oxidase, starch-associated R1, and phosphorylase-L genes. This genetic modification was accomplished without inserting any foreign DNA into the plant genome. French fries from the intragenic potatoes also contained reduced amounts of the anti-nutritional compound acrylamide while, unexpectedly, displaying enhanced sensory characteristics. Processed potato tuber texture is an important trait that influences consumer preference, a detailed understanding of tuber textural properties at the molecular level is lacking. Tuber pectin methyl esterase activity is a potential factor impacting on textural properties. Expression of a gene encoding an isoform of pectin methyl esterase (PEST1) was associated with cooked tuber textural properties. Ross et al. (2011) changed the texture of potato by engineering pectin and thus changed the texture of potato. They used transgenic approach to investigate the impact of the PEST1 gene. Antisense and overexpressing potato lines were generated. In overexpressing lines, tuber pectin methyl esterase activity was enhanced by up to 2.3-fold, whereas in antisense lines, pectin methyl

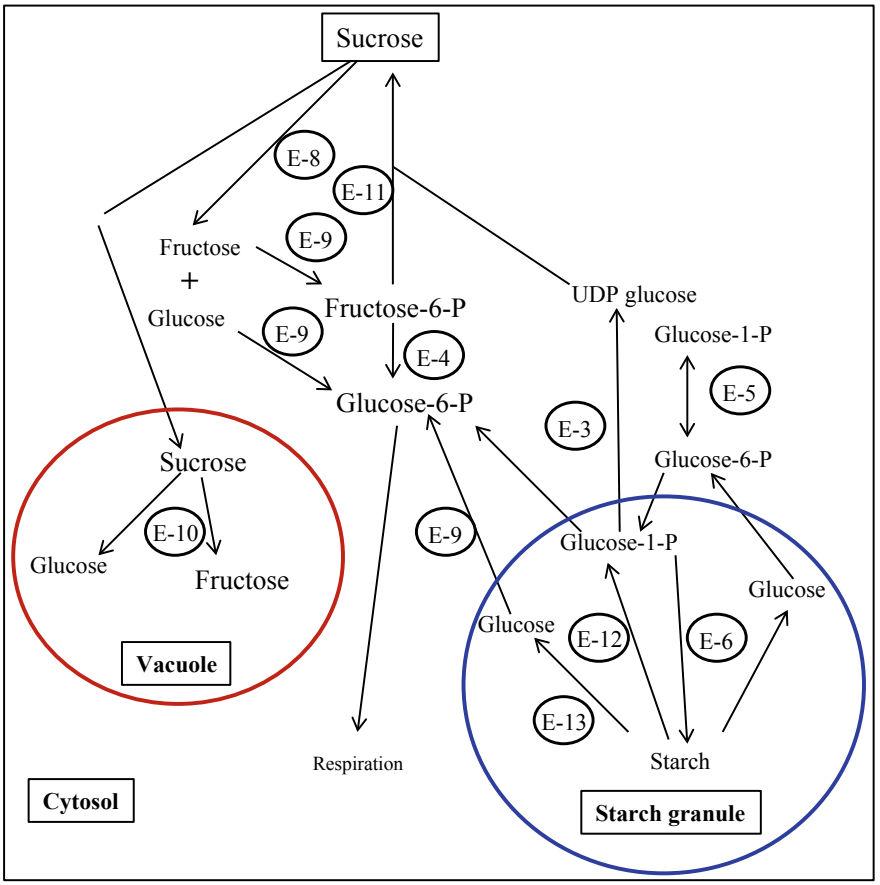

Fig. 15.12 Carbohydrate metabolism in stored potato tubers. Enzymes: (E1) sucrose synthase; (E2) fructokinase; (E3) UDP glucose pyrophosphorylase; (E4) phosphohexose isomerase; (E5) phosphoglucomutase; (E6) ADP glucose pyrophosphorylase; (E7) starch synthases, branching enzymes; (E8) neutral invertase; (E9) hexokinase; (E10) acid invertase; (E11) sucrose phosphate synthase; (E12) starch phosphorylase; (E13) amylases, debranching enzymes. Partially adapted from Dale and Bradshaw (2003)

esterase activity was decreased by up to 62%. Pectin methyl esterase isoform analysis indicated that the PEST1 gene encoded one isoform of pectin methyl esterase. Analysis of cell walls from tubers from the overexpressing lines indicated that the changes in pectin methyl esterase activity resulted in a decrease in pectin methylation. Analysis of processed tuber texture demonstrated that the reduced level of pectin methylation in the overexpressing transgenic lines was associated with a firmer processed texture. Thus, there was a clear link between pectin methyl esterase activity, pectin methylation, and processed tuber textural properties (Ross et al. 2011). Potato polyphenol oxidases are the enzymes responsible for enzymatic browning reaction observed in impacted, damaged, or sliced tubers. These oxidative deterioration reac-

tions alter the organoleptic properties of food and greatly affect potato tuber quality. Llorente et al. (2011) silenced the *PPO* gene in transgenic potato which reduced the enzymatic browning and enhanced the shelf life of potato.

15.5 Future Prospects

Malnutrition is the most chronic and pressing agricultural and human health problem of the twenty-first century. Potato being an important constituent of our diets is expected to play vital role in tackling this serious malnutrition problem. Therefore, improvements in the nutritional quality of potato would have most visible positive impact. For achieving this, full potential of biotechnological tools must be put in use in association with conventional plant breeding programs with the sole aim of bio-fortifying potatoes with superior nutritional levels. The genomic resources need to be continuously enriched to have deeper insights for identifying key molecular regulators which can be utilized through biotechnological approaches in potato with the aim of developing nutritionally superior cultivars. Present mechanisms put in place for evaluation and recommendation for commercial application of the genetically engineered superior potato, and other crops need to be streamlined so that the real benefit of the biofortified nutritionally superior food can reach the consumers.

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Chapter 16
Role and Applications of Bioinformatics in Improvement of Nutritional Quality and Yield of Crops

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Abstract Bioinformatics has the major role to play in decoding of the genomes of plants and animals. Bioinformatics is making progress in each and every field of life sciences, and similarly, the field of crop improvement has also been influenced by it. Bioinformatics allows capturing, managing, analyzing, and integrating the huge amount of metabolomics, genomics, and proteomics data enabling its efficient interpretation by the users. Bioinformatics makes available data and various tools to every individual, company, or industries so as to increase nutritional value and yield of crops. Detection of complex protein–protein interactions, modeling the protein structures, and unraveling the high-resolution genetic and physical network in plants can also be easily accomplished using *in silico* studies. This book chapter basically reviews the different role and applications of bioinformatics in plant breeding, gene network analysis, and molecular marker-assisted crop improvement techniques.

Keywords Bioinformatics · Plant breeding · Microarray · Gene network · Molecular markers and QTL

16.1 Introduction

Let it be the sequence data of animals, humans or plants, bioinformatics has the major role to play in decoding of their genomes. Bioinformatics is making progress in each and every field of life sciences, and similarly, the field of crop improvement has also been influenced by it. Scientists have succeeded in increasing the levels of

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iron, vitamin A, and other micronutrients through gene transfer in rice. Most prominent example of bioinformatics in improving the nutritional quality of the crop is the development of golden rice that can fight against vitamin A deficiency in humans. This new variety of rice can help in reducing the occurrence of anemia and blindness due to deficiencies in iron and vitamin A, respectively (Paine et al. 2005). Crop yield can be increased by designing plants based on understanding the regulatory network and gene function involved in growth, stress tolerance, and development. This can be achieved by using bioinformatics approaches such as comparative genomics. Comparative genomics identifies the biological properties of each species that accelerates the functional analysis of gene and its discovery. Like in *Theobroma cacao* (cocoa) a raw material for chocolate, DNA fingerprinting is used to recognize a seed of good flavor and higher quality. Bioinformatics allows capturing, managing, analyzing, and integrating the huge amount of data enabling its efficient interpretation by the users. In the area of genomics, it has the role in gene location, evolutionary studies, and identification of transcription regulatory sites in the genes. Detection of complex protein–protein interactions, modeling the protein structures, and unraveling the high-resolution genetic and physical network in plants can also be easily accomplished using *in silico* studies (Basantani et al. 2017; Mochida and Shinozaki 2010). The explosive growth of biological data due to the development in genomic and proteomic approaches over the last few years was handled through bioinformatics in all possible ways, e.g., storing the data efficiently in databases, designing the computational tools to analyze the stored information, designing the visualization tools, and integrating the related information from various resources, so that the users can draw the meaningful conclusions from the huge data sets (Ballabh et al. 2017). Bioinformaticians are involved in writing and running the software programs that use algorithms from artificial intelligence, soft computing, graph theory, and computer simulation. All these above-mentioned bioinformatics services have an important role to play in molecular plant breeding techniques to improve crop yields and nutrition. Information of full genome sequence of plants and agriculturally important microorganisms available in the different databases can be analyzed to identify the key genes to enhance the yield and nutritive content of the crops. The genetic architecture of microorganism and pathogens can be analyzed to check the mechanism of action affecting the host plant by using metagenomics and transcriptomics approach. This could help to generate the pathogen-resistant crops. Bioinformatics tools can also be used for the identification of miRNA or siRNA in plants as these are the potential targets for the crop improvement. Moreover, marker-assisted selection (MAS) is also in use in crop improvement programs since the improvement in sequencing techniques. This book chapter basically reviews the different roles and applications of bioinformatics in plant breeding, gene network analysis and molecular marker-assisted crop improvement techniques.

16.2 Bioinformatics in Plant Breeding

Plant breeding means altering the genetic makeup of plants to produce the desired characters. It can be used to improve the nutritional quality of crops or increasing the yield. Sequence data of the plants can be used to identify the targets for plants breeding by understanding the organization of the whole genome, structure of the genes, and expression pattern of the genes. Out of these, transcriptomics is the focus of attention for most of the plant breeders. Transcriptomics means to study the complete set of RNA transcripts produced by the genome of an organism at a particular time under definite conditions (Vilanova et al. 2012; Atanassov et al. 2014).

This sub-category of omics allows identification of differently expressed genes in different cells, at different times, and different conditions. The microarray is a high-throughput technology to quantify the gene expression data to draw the functional inferences about different genes simultaneously. The microarray experiments produce the number of data points that require the computational interpretation for meaningful data analysis. There are number of tools and Web-based resources available for analysis of microarray data that utilize the different statistical packages like SAS, MATLAB, and R for data analysis. The information stored in various public and private databases also needs to be integrated for interpretation of huge microarray data (Vassilev et al. 2005).

16.2.1 Software for Microarray Data Analysis

The series of images are obtained in microarray experiments. The software is used to analyze the images to reveal the intensity of the spots which quantify the expression of transcripts at each spot. The obtained information is normalized and statistically analyzed using various commercial or freely available software. These software can contribute to image analysis to obtain the gene list, ontology, and gene pathways (Koschmieder et al. 2012). Some of the examples of such software are:

Microarray Analysis, Retrieval, and Storage System (MARS)
MARS is a MIAME supportive (minimum information about a microarray experiment) suite for analyzing, retrieving, and storing multi-color microarray data. This system incorporates a laboratory information management system (LIMS), a sophisticated user management system, as well as quality control management. It is fused into an analytical pipeline of microarray image normalization, analysis, mapping of gene expression data onto biological pathways, and gene expression clustering. The inclusion of Microarray Gene Expression Markup Language (MAGEML) and the use of ontologies permit an export of studies to databases and other public repositories accepting these documents stored in MARS. It is available for both academic and non-commercial use (Maurer et al. 2005).

BioArray Software Environment (BASE)
BASE provides a MIAME-comprising application, which is designed for storing microarray experiments related to the information generated by microarray laboratories. It is a local data repository for multi-users that have various elements like LIMS for array and biomaterial production, annotation outline for analysis, user interface for Web browser and integrated tools like GenePattern and MultiExperiment Viewer (MEV) (Vallon-Christersson et al. 2009).

New version of BASE provides the users with more efficient analysis tools and information management system (INS). The information by INS is collected from biosources, and raw data is labeled and analyzed comprehensively. Annotation can be done for all items in BASE and data post-annotation can be utilized for experimental factors. Then the microarray experimental data and its annotations are stored by BASE regardless of the provided data format.

Chipster
Chipster (http://chipster.csc.fi/) is a collection of user-friendly visualization and analysis tools for microarray data understanding. It consists of a vast assembly of reference genomes and more than 400 analysis tools. The automatic analysis workflow can be shared and saved by users, and the data can also be visualized interactively using the built-in genome browser. For the actual analysis, the chipster's client software connects to computer server and uses Java, which is installed automatically. It is open-source and the server environment is available as a virtual machine image. A user account is required for Chipster running on CSC's server.

The different data types like array comparative genomic hybridization (aCGH), gene expression, and miRNA can be integrated and analyzed by users. Along with the interactive visualization and large analysis functionality, the users can create new gene list based on the selection of data points. One of the important advantages is that the performed analysis steps can be saved by users for future use, and the automatic workflow can also be shared with other users. Chipster is an easily extendable and versatile platform which can be used for sequencing data, proteomics, and microarray (Kallio et al. 2011).

Gene Set Enrichment Analysis (GSEA)
GSEA is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states (e.g., phenotypes). It is easier to run the analysis on GSEA software and review its results, allowing the user to focus on interpreting the analysis results (Subramanian et al. 2005).

The steps for running an analysis are as follows:

1. Data files preparation:

   - Expression dataset file (res, gct, pcl, or txt)
   - Phenotype labels file (cls)
   - Gene sets file (gmx or gmt)
   - Chip (array) annotation file (chip)

2. Load your data files into GSEA.
3. Set the analysis parameters and run the analysis.
4. View the analysis results.

16.2.2 Microarray Databases

There are number of databases available for hosting the data of hybridizations and microarray analysis process depends on these databases with support of various software.

ArrayExpress
ArrayExpress (www.ebi.ac.uk/arrayexpress) is a database of high-throughput functional genomic data and is publically available. It consists of two parts namely—ArrayExpress Data Warehouse, which stores data selected from repository of gene expression profiles and consistently reannotate and the ArrayExpress Repository, it is a microarray data archive and support MIAME. By using experiment attributes like species, author, keywords, accession number, array platform or journal, the archived experiments can be queried. Gene expression profiles can be visualized and can be queried by gene properties and names, such as Gene Ontology terms. ArrayExpress is a rapidly growing database, having data from >1,500,000 individual expression profiles, and >50,000 hybridizations. MAGE-ML and MIAME community standards are supported by ArrayExpress, and more recently MAGE-TAB a spreadsheet-based data exchange format was proposed (Athar et al. 2019).

Gene Expression Omnibus (GEO)
GEO (http://www.ncbi.nlm.nih.gov/geo/) created in 2000 freely distributes and archives functional genomics and high-throughput gene expression data in an international public repository. It is a resource for gene expression studies and accepts high-throughput data from many other data applications including those that examine genome–protein interactions, genome methylation, and chromatin structure. Community-derived reporting standards are supported by GEO that specifies the provision of several critical study elements including descriptive metadata, raw data, and processed data. GEO provides the data from numerous gene expression studies as well as access to different web-based strategies and tools. It enables the users to analyze, visualize and locate the data relevant to their specific interests with ease (Barrett et al. 2013).

Center for Information Biology gene EXpression database (CIBEX)
CIBEX (http://cibex.nig.ac.jp) is a public repository developed by MGED society and is a data retrieval system supporting MIAME, for storing and comparing microarray data produced in experiments at different laboratories. CIBEX serves to store a wide

range of gene expression research data from high-throughput experiments, including microarray-based experiments measuring serial analysis of gene expression (SAGE) tags, mRNA, and mass spectrometry proteomic data (Ikeo et al. 2003).

16.3 Gene Network Analysis

The analysis of networks or pathways is important for designing of agrochemicals for selected targets in the pathways. The pathway databases are the result of intense biological research to reveal the series of molecular events and could be of real use by genetic engineers, e.g., if the pathways for the synthesis of essential amino acids are known to scientists than this information could be used by them for improvement in nutritional quality of food crops through genetic engineering techniques. Though plant pathway databases are lagging behind, still the researchers of the plants are exploiting the available network data and their visualization tools for their studies (Tien Lea et al. 2016; Sucaet and Deva 2011). Some of the examples of pathway databases are:

Kyoto Encyclopedia of Genes and Genomes (KEGG)
KEGG (http://www.genome.ad.jp/kegg/) knowledge base is daily updated for gene function systematic analysis, linking genomic information with higher-order functional information. It also includes a GENE's database with up-to-date annotation of gene functions for some partial genomes and gene catalog of all completely sequenced genome. The PATHWAY database stores higher-order functional information, cellular processes like cell cycle, metabolism, signal transduction, and membrane transport are graphically represented. The ortholog group table in PATHWAY database stores information about conserved sub-pathways (pathway motifs), which are useful in predicting gene functions and often encoded by positionally coupled genes on the chromosomes. Information about enzyme reactions, chemical compounds, and enzyme molecules is stored in LIGAND the third database in KEGG. In KEGG, genome maps are browsed, two genome maps are compared, and expression maps are manipulated, as well as computational tools for graph comparison, path computation, and sequence comparison is done by Java graphics tools (Kanehisa et al. 2019).

MetaCyc
MetaCyc (http://ecocyc.org/ecocyc/metacyc.html) is a curated database of experimentally elucidated metabolic pathways from all domains of life. From 3009 organisms, MetaCyc contains 2722 different pathways. It contains information about associated metabolites, genes, enzyme, reactions as well as pathways involved in both primary and secondary metabolisms. MetaCyc aim is to catalog the universe of metabolism by storing a representative sample of each experimentally elucidated pathway. Information from multiple literature sources is integrated for a given entry in MetaCyc and is a review-level database. After the experimental determination of

pathways, these are labelled with the species in which they occur based on literature references and stored in the MetaCyc. The database has various applications like biochemistry education, genomes pathway analysis, and metabolic engineering. The graphical user interface for the pathway tools is used to query MetaCyc; it also provides a wide variety of visualization tools and query operations. It is available for installation as a set of flat files and as a binary program for the Sun workstation and for PC (Zhang 2005; Caspi et al. 2014).

AraCyc

Biochemical pathways of *Arabidopsis thaliana* can be visualized by AraCyc (Mueller et al. 2003). Pathway tools developed at SRI by Peter Karp's group support AraCyc. Originally, MetaCyc was used as a reference database to computationally predict AraCyc for the sequenced *Arabidopsis* genome. The non-Arabidopsis pathways were removed and predicted pathways were manually validated. It contains a mixture of information usually extracted from computational prediction and peer-reviewed literature. AraCyc is released semi-annually. With each release, a Pathologic Software Report as well as summary of the database content, is made available (Zhang 2005; Mueller et al. 2003).

Plant Reactome

Plant Reactome (http://plantreactome.gramene.org/) is developed as a part of the Gramene project and is curated, open-source, and free plant pathway database. This database supports to genome analysis, systems biology, education, genome annotation, modeling, and basic research by providing bioinformatics tools for the interpretation, analysis, and visualization. Plant cell structural framework is employed by Plant Reactome to show developmental, transport, signaling, and metabolic pathways. Manual curation of molecular details is done for pathways in these domains and rice (*Oryza sativa*) is considered as reference species. One thousand one hundred seventy-three proteins associated with 1025 reactions, 222 rice pathways, 256 literature references, and 907 small molecules have been curated to date. Various components of the database can be searched and browsed by users. The users can also visualize, analyze, or upload their omics datasets within the database. The information from this database can be accessed in the form of various standardized pathway formats, such as BioPAX and SBML (Naithani et al. 2017).

Cytoscape

Cytoscape integrates molecular states into a unified conceptual framework and high-throughput expression data with biomolecular interaction networks. It is applicable to any system of molecular interactions and components but is most powerful when used in conjunction with large databases of DNA-protein, genetic interactions, and protein–protein interactions that are increasingly available for model organisms and humans. Software Core provides basic functionality to visually integrate the network with other molecular states, expression profiles and phenotypes; to query the network and layout; and to link the network to databases of functional annotations.

By straightforward plug-in architecture, the Core is extensible, which allows rapid development of additional computational features and analyses (Shannon et al. 2003).

GenMAPP

GenMAPP application is freely available; it is designed to visualize genomic data and gene expression on maps representing a grouping of genes and biological pathways. GenMAPP is integrated with various programs to perform a global analysis of genomic data; the MAPPFinder module of GenMAPP can extract the gene expression in the context of thousands of Gene Ontology Terms and hundreds of MAPPs pathway. It exports the archives of genomic/expression data to the Web and imports lists of the proteins/gene to build new MAPPs (MAPPBuilder).

GenMAPP-CS is a cross-platform program composed of multiple plug-ins within the program Cytoscape. GenMAPP-CS interfaces with WikiPathways to obtain pathways by reading the GPML format of the pathway files. GenMAPP-CS also uses species gene databases built through the BridgeDB project, which also provides databases for WikiPathways and PathVisio. GenMAPP-CS provides many of the same features as GenMAPP 2.0, with many additional and improved methods for gene and sub-gene analyses (e.g., GO-Elite, ClusterMaker, APT Affymetrix array normalization, GEO import advanced custom visualization methods, and other methods available from Cytoscape plug-ins). GenMAPP-CS has an easy to use graphical user interface, designed for the bench laboratory biologist (Salomonis et al. 2007).

16.4 Molecular Markers

Molecular markers follow a Mendelian pattern of inheritance and are defined as variations in DNA sequences on the homologous chromosome of two distinct individuals at the identical position. Past two decades, the whole plot of life sciences has been remodeled because of approach of molecular markers. The molecular markers based on DNA are a versatile tool in the field of physiology, genetic engineering, taxonomy and embryology, etc. (Schlötterer 2004). Ever since the improvement of molecular markers, these are uniformly being revised to intensify the efficiency and to bring about self-regulation in the method of genome analysis. The development of polymerase chain reaction (PCR) was a milestone in this effort and demonstrated as an innovative process that brought about a new form of DNA profiling markers. This facilitated the development of marker-based gene tags, genetic mapping, map-based cloning of agronomically important genes, genetic diversity studies, phylogenetic analysis, and marker-assisted selection of desirable genotypes, etc. (Joshi et al. 2000).

16.4.1 Quantitative Trait Loci (QTL) Mapping

In plant breeding, one of the most challenging tasks is to associate the development of traits that show a continuous range of values. Quantitative traits also known as complex or multifactorial or polygenic traits are regulated by many genes and are genetically important traits associated with some form of the disease, quality, and yield. QTLs are genetic factors that are responsible for a part of the observed phenotypic variations for a quantitative trait. QTL mapping is the process of identifying the genomic regions associated with these traits, i.e., to conduct QTL analysis and creating linkage maps. DNA markers are used for location and identification of QTL or polygenes. DNA markers are tested throughout the genome to detect their association with QTL. The analysis of QTL can be done in progeny based on the principle that markers and genes are segregated by chromosome recombination during meiosis. For QTL mapping, an association between genotype and phenotype of markers is detected, and it depends on the linkage disequilibrium.

Objectives of QTL mapping:

The main objective is to minimize the occurrence of false positives (i.e. Type I error—declaration of an association between a QTL and marker when there is none) and QTL detection.

- Identification of genome regions that affect the trait of interest;
- Analysis of the effect of QTL on trait;
- Identifying the effect of specific region affecting the variation of the trait;
- Identification of additive or dominant gene action associated with the QTL;
- Identification of alleles associated with a favorable effect.

From an inter-sub specific population of *Lens culinaris* ssp. *orientalis* x *Lens culinaris* ssp. *culinaris*, Duran and Vega (2004) developed a QTL map for pod dehiscence, seed diameter, plant height, and number of shoots. Humphry et al. (2003) and Chaitieng et al. (2002) found one QTL in *Vigna* species responsible for powdery mildew resistance while Young et al. (1993) identified three QTL in mungbean associated with powdery mildew resistance. Six AFLP-derived QTLs were identified by Sholihin and Hautea (2002) associated with two traits (leaf stress rating and leaf relative water content) that can be used to measure the drought tolerance.

16.4.2 Tagging of Disease Resistance Genes

The most cost-effective and efficient way of controlling bacterial blight is the use of resistant cultivars. The primary step toward crop improvement by the map-based cloning and marker-based selection of resistance genes through the discovery of molecular markers closely linked to genes of interest. Xa-1, Xa-2 and bacterial blight resistance genes have been tagged with RFLP markers (Yoshimura et al. 1992). With

the help of PCR-based RAPD markers (from DNA samples random fragments of DNA are amplified using arbitrary, short primers), many more resistance genes can be tagged. DNA markers tightly associated with the genes of interest can be identified by the use of near-isogenic lines (NILs). NILs are produced by repeated backcrossing and selection for a target gene. Thus, in the genetic history of the chronic parent, a NIL carries a target gene with a small fragment of the donor parent genome. For bacterial blight resistance genes, a number of NILs have been developed (Ogawa et al. 1988, 1991).

Host resistance is supposed to be an approach of control to the bacterial disease. However, the resistance provided by a single gene may not be secure because it can be overwhelmed by inherent or newly evolved pathogen races. In a cultivar, resistance genes may be coupled ('pyramiding' of resistance genes) and can be useful for generating varieties with longer-lasting resistance and broader resistance spectra (Jennings 1979).

16.4.3 Male Sterility Genes Tagging

For production of heterogeneous seeds, a cytoplasmic male sterile (CMS) system is used as the need for manual emasculation is excluded. CMS is portrayed by anthers producing non-viable pollens without affecting the fertility of female; this characteristic is maternally inherited and is usually linked with rearrangement, editing, and mutations in mitochondrial DNA. RAPD and STS have been used to recognize numerous restorer loci in various crops and molecular study of CMS system is facilitated by DNA markers linked to these loci. The homozygous restorer genotypes can be recognized by these codominant markers after production of restorer lines by backcrossing. Compared to traditional techniques, the restorer lines can be produced in a shorter period in this way. In 2003 RAPD markers linked to male-sterility genes were identified by Souframanien et al. (2003). A unique amplicon of 600 bp was produced by primer in male sterile (A) lines 288A (*C. scarabaeoides* derived) and 67A (derived from *C. sericeus*), which were not present in their respective, maintainers, and putative R lines (TRR 6 and TRR 5). The significant genetic variation based on the similarity index was observed between two putative R lines, donors of male sterility genes, and male sterile lines.

16.4.4 Testing of Hybrid Seed Purity

The hybrid seed characters can be determined by confirming that the desired cross has happened, the required purity has been met by self-pollination within the female parents, and the quality of the product is sufficient. The grow-out test has been the only way from decades to check the hybrid seed purity. But now the purity of F1 hybrids can be tested by using RAPD and RFLP markers. Kumar et al. (2011) tested

the F1 purity of H 86 × EC 520061, Pbc × EC 538408 and Pbc × EC 520061 tomato hybrids by using male-specific markers SSR 306, Ty2 gene, and SSR 218 CAPs gene marker (Kumar et al. 2011).

16.4.5 Gene Pyramiding

In gene pyramiding, multiple genes are determined and included which have attained resistance through autonomous host pathway toward a single pest or to an autonomous microbial/insect pest. The resistance strength can be increased by combining multiple resistance genes into a single species. The strength of resistance can be increased by 50 years if the single gene has never been used in pyramided genes. The numbers of resistance gene that have been successfully pyramided are difficult to confirm through the process of constructing a cultivar. The resistance of plant having three resistant genes is more enduring as compared to the plants with only one resistance gene.

16.4.6 Map-Based Cloning of Genes

The initial step in map-based cloning is to identify the molecular marker lying adjacent to the gene of concern. Starting from a small mapping population to a higher saturated gene map is an essential step to clone a gene. The region around the initially identified molecular marker is saturated with many other markers. Hence, screening of a large number of markers is done to obtain a marker recombining infrequently with the gene of interest. In the next step, the clones that hybridize with markers are isolated by screening of large-insert genomic library (YAC or BAC). Using the chromosomal walking technique, the target gene is searched through two flanking markers are identified which show linkage with the target gene. The aim is to find clones among the set of flanking markers that co-segregate with the gene of interest. The individuals devoid of target genes are introduced with putative clones. The newly cloned gene undergoes characterization by detailed molecular and biochemical analysis in the cases where transgenic has shown to rescue the mutant phenotype.

Pto (bacterial speck disease of tomato resistance) gene of tomato was cloned by map-based cloning technique (Martin et al. 1993). A 251 F2 plant genetic population was screened with DNA probes. *Pto* gene co-segregated with the locus TG 538. Clone PTY538-1 was identified when TG 538 probe was used to screen YAC libraries. PTY 538 transformed susceptible plants were also recovered as resistant phenotype.

16.5 Conclusions

Bioinformatics acts as an interface between the conventional biology studies and information technology and facilitated a considerable breakthrough in biology by not only providing the researchers with loads of genomic information but also the state-of-the-art computational tools for analysis of that information. Bioinformatics has proven itself propitious every time for various discoveries in the field of agriculture, whether it is about the prevention or targeted treatment of diseases, improvement of the nutritional quality of food crops, or increasing the yield of crops. The accurate and cautious effort of plant breeders along with the facilities provided by bioinformatics has led to the notable progress in agriculture, e.g., the introduction of the semi-dwarf wheat, rice crossed a variety of millets, brown rice, etc. Significant rise in the yield of oilseeds and cotton has also been witnessed. All these objectives were achieved by enriching the conventional breeding techniques with molecular breeding techniques, e.g., marker-assisted selection. The *in silico* studies also play an important role in understanding the protein–protein interactions in plants and pathogens. So, the several strategies of bioinformatics with further improvement in them can be exploited further to make more major contributions in the field of agriculture.

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