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Nutrient Biofortification of Staple Food Crops: Technologies, Products and Prospects

Chavali Kameswara Rao[1] *and Seetharam Annadana*[2]

[1] *Foundation for Biotechnology Awareness and Education, Bangalore, India*
[2] *Technology Lead for an MNC, Krishi, 29, CR Layout, Sarakki Main Road, Bengaluru, India*

3.1 Introduction

All through the human history it was recognised that food plays a significant role in sustaining health. '*Let food be thy medicine*', a quote attributed to Hippocrates some 2,400 years ago, was the title of the 2016 Borlaug Dialogue (WFPO 2016). About a 100 years ago, Thomas Alva Edison adverted to the Hippocratean advice in the observation that '*the doctor of the future will no longer treat the human frame with drugs, but rather will cure and prevent disease with nutrition*' (Snopes.com 2015).

The Rome Declaration of the World Food Summit of 1996 defined '*food security*' as existing '*when all people at all times have access to sufficient, safe, nutritious food to maintain a healthy and active life*'. This definition actually reiterates the essentiality of not just '*sufficient food*' but also of '*nutritious food*', to maintain a healthy and active life, highlighting that '*nutritional security*' is an integral component of food security.

For decades now, international organisations such as the Food and Agricultural Organisation (FAO), the World Health Organisation (WHO) and the International Food Policy Research Institute, Washington (IFPRI), have been working hard to motivate governments of different countries on the essential and effective policy imperatives needed to ensure food security to alleviate hunger. In recent times there is more emphasis on the urgent need for ensuring nutritional security as well.

The main thrust of the Rome Declaration, reiterated by the World Summit on Food Security in November 2009 (FAO 2009), was to reduce the number of the world's 1 billion hungry and chronically undernourished people by half (to 500 million), by 2015. This objective was, however, not achieved and 795 million people still remain hungry (FAO 2015, EIU 2016, von Grebmer *et al.* 2016), and one-third of the global population suffers from micronutrient malnutrition (IFPR 2016). The EIU's (2016) Global Food Security Index (GFSI) is based on a unique country-level food-security measurement tool that addresses the issues of availability, accessibility and affordability (see 11. Conclusions and Recommendations) in 113 countries around the world.

Food security would be incomplete without nutritional security. Genetic engineering (GE) has been advocated as the most effective tool to enhance crop productivity and for

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food crop '*biofortification*' (the breeding of genes for nutrients into staple food crops), by Zhu *et al.* (2007), Meyer *et al.* (2008), Hirschi (2009), Beyer (2010), Newell-McGloughlin (2010), Potrykus (2010), von Braun (2010), Bouis *et al.* (2011), Dubock (2013), Rawat *et al.* (2013), Kameswara Rao (2005, 2013, 2015) and Patil and Kameswara Rao (2015), among others. An array of new genetic technologies, as discussed in the later pages of this chapter, are also being developed involving huge time and financial investment, to enhance the nutrient content of crop plants to improve human health.

The urgent need to ensure nutritional security particularly in the developing countries is recognised by the award of 2016 World Food Prize to three scientists honoured for the development and implementation of biofortification, to significantly reduce '*hidden hunger*' (another name for undernutrition and malnutrition) of millions worldwide.

The Green Revolution has done wonders to enhance staple food security in general in many countries, but as the vitamins, minerals and other nutrients are often in inadequate quantities in the foods we consume, our nutritional security is under threat. Nutritional enhancement of crops is now being seen as an important component of the second or the '*Evergreen Revolution*'.

The objective of this chapter is to discuss (a) the background on the biological roles of macro- and micronutrients in sustaining our health, (b) the strategies to enhance their intake, (c) the process of enhancing the quality and levels of nutrients in food crops, (d) the technologies used and being now developed for biofortification, (e) biofortified food crops developed so far, (f) the future prospects of food crop biofortification and (g) recommendations to ensure nutritional security in the developing countries.

3.2 The Concepts of Nutrition and Malnutrition

During the past couple of decades, food, nutrition and health have attracted the attention of the professionals and laymen alike, resulting in voluminous scientific as well as popular literature. Unfortunately, most of the latter has become a banter needing the re-establishment of scientific temper into public education on the issues of food and nutrition. Even those well considered and publicised 'scientifically established' beliefs about the macronutrients and cholesterol have been reversed in recent years as discussed in the later pages, in the context of carbohydrates, proteins and fats. There is also a lot of incorrect understanding of the basic terms and concepts related to food and nutrition. Often publications that deal with issues of nutrition do not indicate the biological roles and need for the nutrients, because of which the non-biologist readers and the policy makers are at loss as to the significance of different nutrients and issues concerning food, nutrition and health. For this reason, background information on diverse concepts and nutrients is provided hereunder and contextually in the later pages of this chapter.

3.2.1 Nutrition, Macronutrients, Micronutrients and Balanced Diets

Nutrition is the process of consuming, absorbing and using nutrients, the chemical substances in the food eaten, needed by the body for growth, development and maintenance of life. When nutrient levels are inadequate, '*nutritional security*' is threatened leading to nutritional deficiency disorders that would cause disease and even death (Beers 2003).

Plants produce the wide range of major and minor nutrients we need but they are unevenly distributed in different species and parts of edible plants used as food. The carbohydrates, proteins and fats are the '*macronutrients*' which are the major energy sources consumed in large quantities. Two thirds of the global population depends on foods derived from cereals, millets and tuber crops for the energy required, but these foods do not provide much of the other nutrients needed. A number of vitamins, amino acids, fatty acids and mineral elements, called the '*micronutrients*' as they are needed in small quantities, are equally important for proper nutrition. Among micronutrients, about 13 vitamins, 9 amino acids, 2 fatty acids and 20 mineral elements, which cannot be synthesised by the human body, should essentially come from food (World Bank 2008). As no single food item contains all the nutrients we need, several diverse foods are needed to constitute a '*balanced diet*' to provide appropriate quantities of both the macro- and micronutrients needed for health.

3.2.1.1 Macronutrients and Energy Requirement

Energy is needed by the biological systems for all metabolic activities and processes. Energy is measured in terms of '*calories*', expressed as '*kilo calories*' (Kcal). While all digested foods provide some energy, the principal energy sources are the '*macronutrients*', the carbohydrates (4 Kcal/g), proteins (4 Kcal/g) and fats (9 Kcal/g), which are interchangeable in proportion to their energy content. The macronutrients supply 90% of the dry weight of the diet and 100%of its energy. *The average minimum energy requirement per day is 1,800 Kcal/day, and the general recommendation in most countries is between 2,100 and 2,700 Kcal/day, depending upon the gender, age, physical activity and state of health*. The energy that is not immediately expended is stored as carbohydrates, usually as starch in plants and glycogen in the liver and muscle in animals. Most of the excess energy is stored as fat which contributes to weight gain.

3.2.1.2 Micronutrients

Of about 2 million chemical compounds known from biological systems, close to 1,00,000 substances have been identified in foods consumed in daily diets. However, only about 300 are recognised as nutrients and only 45 are classified as essential nutrients (Beers 2003). These are the '*micronutrients*' which are essentially needed but in small quantities, from micrograms to milligrams. Vitamins, some amino acids, some fatty acids and some mineral elements which enable the body to utilise the macronutrients constitute the micronutrients (Beers 2003). As sources of energy, carbohydrates, proteins and fats can be interchanged but there are no substitutes to any of the micronutrients. The micronutrients critical to health are discussed in the relevant sections in this chapter.

3.2.1.3 The Balanced Diets

The term '*diet*' denotes the combination of different foods one eats at one or more sittings in a day. The term applies equally to what healthy people consume as much as to people such as the sick, infirm, pregnant or lactating women, infants, young children and so on, who need to consume different kinds of special diets. In order to receive adequate and appropriate nutrition, one needs a '*balanced diet*', which consists of a judicious combination of different foods such as cereals, pulses, vegetables, fruits, dairy products and meats, to provide diverse nutrients. Such a balanced diet enables people to maintain a desirable body weight and composition of the percentage of muscle and fat and to perform their daily physical and mental activities. Many countries have culturally developed diets that have kept their populations in reasonable health over millennia. However, people of low-income groups, particularly in the developing countries, who cannot afford balanced diets suffer most from nutritional deficiencies.

3.2.2 Hunger, Nutritional Security, Undernutrition and Malnutrition

IFPRI (2016) refers '*hunger*' to an index based on component indicators which, taken together reflect deficiencies in calories as well as in micronutrients. Ensuring '*nutritional security*' involves addressing a number of factors simultaneously. Deficiencies in any or all of energy sources (carbohydrate, protein and fats), or essential micronutrients (vitamins and minerals) signify '*undernutrition*' or '*undernourishment*' (IFPRI 2016). The FAO defines undernourishment, as the consumption of fewer than about 1,800 Kcal/day, the minimum that most people require to lead a healthy and productive life. Undernutrition is the result of, (a) inadequate intake of food in terms of quantity and/or quality, (b) poor utilisation of consumed nutrients due to infections or other illnesses, or (c) a combination of these factors (IFPRI 2016). '*Malnutrition*' refers more broadly to both undernutrition (problems of deficiencies) and overnutrition (problems of unbalanced diets), such as consuming too many calories in relation to requirements with or without low intake of micronutrient-rich foods.

The 2016 Global Nutrition Report emphasised that '*malnutrition is sweeping the world, fuelled by obesity as well as starvation*', and that '*44% of countries were now experiencing "very serious levels" of both under-nutrition and obesity*' (IFPRI 2016). Malnutrition has been traditionally associated with children who are starving, but now the term applies equally strongly to the children and adults, both underfed and overfed. The emphasis has spread out from starvation to overfeeding and more particularly inappropriate food intake. As per the nutrition report, millions of people are malnourished because they are overweight, as well as having too much sugar, salt or cholesterol in their blood (IFPRI 2016). In this context, the concept of the '*metabolic syndrome*' needs to be seriously considered.

3.2.3 The Metabolic Syndrome

The '*Metabolic Syndrome*' (or '*the syndrome of insulin resistance*') includes (a) high triglyceride levels (>150mg/dL), (b) low high-density lipoprotein (HDL) cholesterol levels (40mg/dL), (c) high low-density lipoprotein (LDL) cholesterol levels (>100mg/dL), (d) high blood pressure (>130/80mm/Hg), (e) resistance to insulin action, (e) high level of blood sugar (>150mg/dL), (f) an increased tendency to form blood clots, and (g) all of these usually associated with overweight (Beers 2003; NIH 2011). These conditions contribute to and together they greatly increase, the risk of coronary heart/artery disease, stroke, cancer and other related ailments. The metabolic syndrome, prevalent in the affluent population particularly in the Western countries, is related to the life style that includes the quantity and kinds of food consumed. One of the major causes is the over consumption of highly refined food products which are rich in added sugars, starch, fats and sodium, but particularly poor in dietary fibre content. Inadequate consumption of micronutrients greatly contributes to the metabolic syndrome.

There is a general agreement on what effects of ill-health metabolic syndrome causes, but there is a disparity of opinion on what causes the metabolic syndrome in the first place, as the issues involved are complex and research inconclusive. Three major issues are:

a) A significant number of chronic diseases (coronary heart disease and diabetes type II) strongly correlate with obesity, an important factor in metabolic syndrome (CSRI 2013). There is strong evidence that sugar is a possible independent risk factor for metabolic syndrome (Malhotra 2013). Current medical opinion is heavily tilted towards the view that sugar is the leading cause of obesity, diabetes type II or metabolic syndrome, but medical research is yet to confirm this conclusion (CSRI 2013), as even the WHO does not recognise sugar as either a cause or a part of the treatment of obesity or related ailments. A recent report indicates that China has a high percentage of obese people, but added sugar consumption in China is the lowest, seven teaspoons/day as against 40 in the United States and 35 in Mexico (CSRI 2013).
b) An important issue in metabolic syndrome is fat intake and the advice has been to lower it. Clinical trials show that we turn dietary carbohydrates into body fat and so a low-carbohydrate diet is much more effective in lowering the level of saturated fat in our blood than a low fat diet itself (CSRI 2015). Low-fat diets are not really right as they are known to cause a decrease in energy expenditure, unhealthy lipid pattern and increased insulin resistance (Malhotra 2013). Our body makes carbohydrate or fat out of protein under certain circumstances, and so decisions on the intake of these three major energy sources are critical. Decisions should mean intake of adequate quantities, maintaining quality. However, the basic dilemma is between high or low intake of carbohydrates and/ or fats.
c) The more complex issues are '*glycaemic index*' (GI) and '*glycaemic load*' (GL), which may be helpful in planning healthier diets. The GI is a measure of the potential of a gram of carbohydrate containing food, to raise the levels of blood glucose, at the point of 2h after intake. This is compared to the GI value 100, of pure glucose taken as the reference carbohydrate (Foster-Powell *et al.* 2002, MIC 2016). The GI of foods is high- (≥70), moderate- (56-69), or low. The GI compares the potential of foods containing carbohydrate to raise blood glucose and insulin response. This is related to (a) the ease of digestion and release of glucose, (b) the quantity of glucose present in the carbohydrate, (c) the amount of insulin it causes to be released and (d) the balance of glucose that remains in the blood, after insulin has moved a certain amount of glucose into the cells. The GL is calculated by simultaneously considering the GI values of all carbohydrate containing components and the quantity of each component, in a serving of that food (Foster-Powell *et al.* 2002, MIC 2016). Dietary GL is the sum of the GLs for all foods consumed in the diet. GI and GL taken simultaneously, reflect the impact of quality and quantity of carbohydrate in a meal or diet, on blood sugar levels and the state of carbohydrate metabolism of the individual.

Several large-scale observational studies indicate that the long-term consumption of a diet with a high GL is a significant independent predictor of the risk of developing type II diabetes and cardiovascular disease (Foster-Powell *et al.* 2002). Evidence accumulated on that a low GI diet might protect against the development of obesity, colon cancer and breast cancer (Foster-Powell *et al.* 2002). There are extensive data available on GI and GL of a number of common carbohydratic foods and diets (Foster-Powell *et al.* 2002, MIC 2016), which will be useful in guiding the choice of appropriate foods and diets. But in practise, measuring blood glucose levels after 2h of intake of a diet/meal is a better indicator of the state of insulin/glucose balance.

The new shift in fat consumption paradigm that affects carbohydrate consumption too (see Section 3.6.5), would probably necessitate a revisit of the concept of metabolic syndrome.

In the context of metabolic syndrome and food induced disorders, the widely prevalent concept of '*junk food*' needs to be critically examined. While technically junk food is the one which has only calories and no other nutrients (as for example, starch, sugar and alcohol), 'food police' include many other foods, such as pizza, pasta, noodles, burger and so on, as well as some widely and regularly consumed Indian and oriental snacks. These are all traditional foods, with proven nutritional benefits, in the countries of their origin. Some of these foods may contain refined starch but they also contain very considerable quantities of protein, fat and micronutrients, no less than in many recommended diets. An enormous number of youngsters and adults immensely enjoy these foods. Labelling them as junk food does not significantly decrease their consumption but only raises a guilt complex on the minds of the consumers, drastically reducing their enjoyment quotient. This is not to deny the possibility that some of these foods may contribute to the metabolic syndrome, but that an exaggeration of their ill effects bypasses the issue.

3.3 Strategies to Enhance Nutrient Intake and Nutrient Content of Plant Foods

3.3.1 Interventions to Enhance Nutrient Intake

Combinations of a large number of traditional foods and beverages have provided for a reasonably balanced intake of nutrients helping diverse world populations in the maintenance of health for centuries. Nevertheless, on account of changed circumstances and a number of constraints such as awareness, it has now become necessary to simultaneously implement different alternate interventions to ensure nutritional security. The deficiency in the intake of the macronutrients such as carbohydrates, proteins and edible oils is more related to socio-economic conditions, particularly to affordability rather than availability in the markets. The quantitative enhancement of intake of a large number of micronutrients, as for example iodine, iron, zinc, calcium, vitamins A, D, E, and of the B-complex group and so on, needs to be addressed by the following diverse means:

a) ***Commercial fortification:*** Commercial fortification involves addition of nutrients to prepared and marketed foods, beverages and water. Iodisation of salt is one of the most successful examples of large-scale commercial fortification in the developing world, particularly because of its simplicity and low technology costs (Gomez-Galera *et al.* 2010). Though not a nutrient, fluoride in toothpaste and water has prevented dental diseases in millions. Nevertheless, in the case of both iodine and fluoride, there is a considerable unwarranted exposure of certain sections of the global population who do not actually need them. Many processed foods, including bread, packaged cereals, milk, soft drinks and some brands of packaged water, are fortified with vitamins and minerals providing for some additional intake of micronutrients. Products of commercial fortification are highly visible in the developed world but they are controlled by corporate interests and the products are often distant from real needs and benefits (Nestle 2002);

b) ***Supplementation:*** Supplementation of nutrients involves providing them in the form of pills, tablets, capsules or fluid suspensions, either individually or in combinations of several nutrients. Supplementation has the advantage of providing several nutrients simultaneously as in many regions of the world malnutrition often involves more than one nutrient but may result in overexposure from overdosing
c) ***Biofortification:*** Biofortification is the means of enriching staple foods with desired micronutrients, through improved cultivation practises and/or conventional to modern breeding strategies (Zhao and Shewry 2011) and provides macronutrients along with the chosen micronutrients. Dietary supplementation and commercial fortification campaigns are highly successful in the developed world and have significantly reduced the incidence of deficiency disorders and syndromes. In contrast, the developing countries have a far less robust and less reliable food distribution infrastructure, poor governance, and inadequate funding, making such programmes largely ineffective and unsustainable (Darnton-Hill and Nalubola 2002), in the uneducated population sectors and in the rural areas. Hence biofortification has become the preferred means of nutrient enhancement. Enhancing the levels of micronutrients in the edible parts of crop plants adds them directly to the diet and is more likely to succeed as compliance does not involve additional effort or expense.

3.3.2 Technologies for Biofortification

The choice of technologies for biofortification requires ground data on the micronutrient needs of targeted population groups, the interventions in practise, the genes and metabolic pathways that regulate nutrient biosynthesis, the bioavailability of the micronutrients in fortified foods and the impact of traditional and modern food preparation practises on the stability of nutrients in fortified foods.

Biofortification of food crops through the application of fertilisers was suggested as the simplest and inexpensive ways of enhancing nutrient content of crops (Zho and Shewry 2011). Fertigation, the application of soluble elements through irrigation, was also tried. There has been some success through these methods in enhancing the mineral nutrients in a few instances (see Section 3.8), but they did not always yield satisfactory results as the absorption and assimilation of minerals in plants is governed by several factors which cannot be easily controlled (Zhu *et al.* 2007). Besides, there is a considerable wastage of the applicants as more is taken by the soil than the plants.

There has been only a limited success for biofortification through conventional breeding which involves human selection out of extensive natural and induced variation (see below). The products are mostly related to mineral content and very few other nutrients (Patil and Kameswara Rao 2015). As the need is for heritable but not environment influenced trait enhancement and as conventional breeding techniques have been either inadequate or even impossible to adopt in the case of many crops, the current biofortification strategy is developing nutrient enriched staple crops through an array of genetic engineering (GE) technologies to enrich the nutrient content of crop plants to improve human health.

The tools of GE are (a) elegant as they seamlessly combine several bioscience techniques, (b) predictable as we can clearly determine the kind of change being introduced, (c) precise as the gene of interest can be inserted at a specific position on a specific chromosome and (d) trackable as we can map back and forth the genes and the changes they cause (Kameswara Rao and Seetharam 2014).

GE crops are commonly known as 'GM' (genetically modified) crops, which is an imprecise term as all products of agriculture and animal husbandry of 10,000 years were all genetically modified, developed through conventional breeding strategies. They were not randomly bred but developed by taking advantage of variation arising out of (a) natural or induced mutations and (b) natural or induced hybridisation, and by a conscious human choice of beneficial traits (Kameswara Rao 2015).

The precise and very useful modern GE technologies involve heavy time and financial costs and rigorous biosecurity regulatory procedures and in some countries there has also been very rampant and persistent activism against GE crops (Kameswara Rao 2015). Nevertheless, modern technologies are essentially needed in biofortification strategies. Several scientists support biofortification through crop GE as an effective means of providing balanced diets to alleviate malnutrition (Zhu *et al.* 2007, Meyer *et al.* 2008, Hirschi 2009, Newell-McGloughlin 2010, Beyer 2010, von Braun 2010, Bouis *et al.* 2011, Dubock 2013, Rawat *et al.* 2013, Patil and Kameswara Rao 2015). As conventional approaches can be useful in certain situations and have produced health promoting combinations of carbohydrate components in wheat, maize and barley mutants (see Section 3.4), they should also be used along with GE protocols (Patil and Kameswara Rao 2015).

3.3.3 Common Genetic Engineering Technologies

A number of diverse protocols have been in use for long to develop various GE products in medicine and agriculture being currently commercialised for over two decades. The GE technologies used in crop biofortification are indicated contextually in the body of this article, while the different technologies are briefly introduced here.

a) ***Recombinant DNA protocols***: GE is predominated by the 'recombinant DNA' (rDNA) protocols, which facilitate the insertion of genes from any organism into the genomes of any other organism irrespective of the degree of genetic relationship, resulting in 'transgenic organisms'. However, GE may also mean addition or removal of genes, enhancement or suppression of gene expression, or somatic hybridisation;
b) ***Gene stacking***: In the first generation transgenic products only one trait/gene was addressed, but soon stacking (or pyramiding) two genes, as, for example, pest tolerance and herbicide tolerance, in the same transgenic, has become routine. Even in some products developed through gene silencing two traits, as for example potato variety with low browning and low acrylamide, were addressed. The three vitamin corn with five stacked genes (Naqvi *et al.* 2009) is a product of intense nutritional significance (see Section 3.7). Gene stacking is a means of addressing more than one problem simultaneously in a single crop variety and can be immensely beneficial. De

Steur *et al.* (2012) estimated the potential health benefits and cost-effectiveness of gene stacked multi-biofortified rice with enhanced pro-vitamin A, zinc, iron and folate concentrations, in China. Using the 'disability-adjusted life year' (DALY) framework, the current annual health burden of the four micronutirents in China was estimated to be 10.6 million DALYs. The multi-biofortified rice, which does not yet exist, is calculated to reduce the DALYs up to 46% (De Steur *et al.* 2012) and would save to costs around US$ 2 per DALY as against US$16 otherwise;

c) ***Gene silencing*** (GS): Gene silencing is a means of suppressing the expression of targeted genes, occurs naturally in all organisms. In biotechnological GS, synthetic anti-sense oligonucloetides or ribozymes (the non-protein enzymes) or interference by RNA (RNAi) are the key elements. These antisense elements bind to the targeted complementary nucleotide sequences in the genome of the recipient organism and prevent the expression of the concerned genes. When the GS elements come from a species different from the recipient species, which is most often the case, the product is transgenic, as it involves rDNA procedures. When the elements come from the same species it is cisgenic. The oliginucleotides and ribozymes are widely used in biomedical research, while transgenic RNAis are the most common tool in crop plant transformation.

   GS has been quite useful in the control of viral diseases and even a few crop pests. Antisense elements were used to design products with desired nutrient composition to enhance their health benefits as exemplified by, (a) the changing of the relative proportions of starch components amylose and amylopectin in wheat and potato (see Section 3.4), (b) modifying levels of proteins and amino acids (see Section 3.5), (c) modifying levels of fatty acids (see Section 3.6), and (d) reducing levels of allegens and antinutritional compounds (see Section 3.10). There are also other products with health implications developed through GS such as, (a) significantly reduced levels of major steroidal glycoalkaloids in potato to make them safer to sensitive individuals (McCue *et al.* 2003), (b) a genetically decaffeinated coffee (Ogita *et al.* 2003), (c) reducing levels of polyphenol oxidases to prevent enzymatic browning of apples and potatoes (USFDA 2015a,b), (d) reducing the levelsof asparagine and reducing sugars to curtail the potential for the formation of acrylamide in potatoes upon heating (USFDA 2015b), or (e) a tearless onion (Kamoi 2008, Eady *et al.* 2008)

d) ***The '-omics' protocols***: The benefits of genome data have been greatly enhanced by a series of analytical protocols developed by molecular biologists and bioinformaticists, collectively called the '-omics' series. These do not necessarily lead to new products but provide for a platform for the evaluation of the safety of the GE products. Some common examples are (a) genomics (the study of genomes, with several subareas such as cognitive, comparative, functional and personal), (b) proteomics (analysis of the entire complement of proteins in an organism, with subareas of structural, functional, immunological and nutritional), (c) glycomics (study of the carbohydrate profiles), (d) metabolomics (chemical fingerprinting of residual small molecules), and (e) metabonomics (metabolic responses to pathophysiological stimuli or genetic modification) (Kameswara Rao 2015). Transcriptomics, the study of the set of all RNA molecules, is actually a part of genomics. The -omics tools are complex, sophisticated and require technical expertise, extensive instrumentation and heavy time and financial inputs, but are likely to provide a better understanding of global effects of GE on metabolites, enzyme activities, and fluxes (Newell-McGloughlin 2010) and are often essentially needed to obtain comparative biochemical profiles of transgenics and their isogenics. More importantly the -omics protocols help in identifying the quantitative and qualitative status of nutritionally significant compounds in a food crop, the genes that are involved in their synthesis, and the isolation and extraction of those genes for developing GE crops with higher quantitative and/or qualitative nutritional potential. The -omics protocols should be employed on a case by case basis. Often they are insisted upon to evaluate substantial equivalence of isogenics and transgenics, and in most cases this is wholly unnecessary and waste of both time and money (Kameswara Rao 2015).

3.3.4 Alternative Genetic Engineering Technologies

A large number of rDNA and other products in medicine, environment and agriculture, possible to develop only through GE, are now widely commercialised globally. Nevertheless, in many countries these products and those in development have been facing the severest opposition from antitech activists, not necessarily on genuine concerns of biosafety or environmental safety. Products of GE are being subjected to very intricate biosecurity regulatory evaluation that imposes heavy time and financial costs. These issues prompted scientists to develop alternative GE technologies which are (a) more precise and fast, and (b) do not leave any DNA from any other (donor) organism in the improved crop/product, and so hopefully do not attract regulatory oversight as discussed in detail by Kameswara Rao (2015). The following are among the more important of these tools:

a) ***Metabolic engineering:*** involves a redirection of one or more enzymatic or other reactions to suppress or enhance the production of existing compounds or produce new compounds, through a genetically mediated modification of the enzymatic, transport, and/or regulatory functions of the cell compounds (Newell-McGloughlin 2010). Research to improve the nutritional quality of plants has been restricted by a lack of basic knowledge of plant metabolism and the challenge of resolving complex interactions of thousands of metabolic pathways (Newell-McGloughlin 2010). Significant progress has now been made in elucidating many plant pathways involving a large number of metabolites with nutritional roles and in the use of cloned genes to engineer plant metabolism. A judicious combination of both traditional and modern genetic modification techniques is needed to metabolically engineer plants to achieve desired levels of qualitative and quantitative enhancement of compounds of significance in human nutrition. Many examples of metabolic engineering that enhanced nutrient levels in plant foods are cited contextually in this article;
b) ***Direct multiple gene transfer:*** Multiple-transgene direct DNA transfer that simultaneously introduces all the components required for the expression of complex recombinant macromolecules into the plant genome was demonstrated in rice by Nicholson *et al.* (2005). Engineered minichromosomes, which segregate independently of the host chromosomes as demonstrated in maize (Carlson *et al.* 2007), can be used simultaneously to transfer and to express stably multiple sets of genes (Houben *et al.* 2008)

c) ***Synthetic proteins and nucleotides:*** Synthetic storage proteins were designed using the standard nucleotide combinations in sweetpotato and cassava (see Section 3.5). More interestingly, the semi-synthesis of a novel *Escherichia coli* with six nucleotide bases that include a fully functional synthetic pair d5SICS-dNaM (denoted as X and Y), in addition to the classic two pairs (Malyshev *et al.* 2014) has opened up new possibilities in synthetic biology with implications for nutrient enhancement in plant foods in the near future.

3.3.5 Recent Genetic Engineering Technologies

A set of new technologies, collectively called gene editing technologies (GET), are causing waves in plant biotechnology as they facilitate quick editing of several genes with either mutational, cisgenic or transgenic approaches, making GE much easier. As products of these tools do not contain any extraneous DNA, the hope is that they do not attract any regulatory oversight (Wolt *et al.* 2016), as already indicated (Waltz 2016). The following GETs protocols allow modification of DNA sequences at precise locations:

a) ***Engineered Meganucleases*** **(EMNs):** EMNs, also known as homing nucleases, are double-stranded DNAses that target large recognition sites and promote efficient gene targeting through double-strand break-induced homologous recombination, a pathway to repair DNA double-strand breaks. Double-strand breaks are important for site-specific mutagenesis in that they simulate the cell's natural DNA-repair processes. EMNs, derived from microbes and modified to cause double-strand breaks, are ideal scaffolds to engineer enzymes that accurately cleave DNA and induce recombination (Molina *et al.* 2011). EMNs were used to induce stacked herbicide tolerance and insect resistance in cotton (D'Halluin *et al.* 2013) and male sterility in maize (Djukanovic *et al.* 2013).
b) ***Zinc Finger Nucleases*** **(ZFNs):** ZFNs are a class of synthetic meganucleases engineered as DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations followed by gene modification during subsequent repair (Urnov *et al.* 2010). ZFNs serve as highly specific genomic scissors. ZFNs facilitate rapid disruption or integration into any loci and the resultant mutations are permanent and heritable. In plants ZFNs were used to induce herbicide tolerance in maize (Shukla *et al.* 2009).
c) ***Oligonucleotide Mediated Mutagenesis*** **(OMM):** OMM is induction of site-specific mutations with chemically synthesised oligonucleotides with homology to the target site (Wolt *et al.* 2016). Herbicide tolerance was induced through OMM in maize (Zhu *et al.* 1999) and rice (Okuzaki and Toriyama 2003).
d) ***Transcription Activator-Like Effector Nucleases (TALENs):*** TALENs are programmable nucleases comprised of the DNA-binding domain of xanthomonas-derived effectors. Zhang *et al.* (2013) optimised TALEN methods using tobacco (*Nicotiana tabacum*) protoplasts, which should be useful for targeted modification of cells from diverse plant species. TALENs can be engineered to bind practically any desired DNA sequence, so that when combined with a nuclease, DNA can be cut at specific locations. The ability to create a valuable trait in a single generation through target modification of a gene family demonstrates the power of TALENs for

genome engineering and crop improvement (Huan *et al.* 2014). TALEN protocols have been used to induce resistance to bacterial blight in rice (Li *et al.* 2012), several mutations in barley (Wendt *et al.* 2013) and to improve the quality of soybean oil (Huan *et al.* 2014).

e) ***Clustered Regularly Interspaced Short Palindromic Repeats*** **(CRISPR/Cas9):** CRISPR/Cas9 is a system of programmable nucleases composed of bacterially derived endonuclease (Cas9) and a single-guide RNA (sgRNA). It is a means to create breaks in double-stranded DNA at specific genome locations, to mutate, replace or add new genes at those locations (Kuzma *et al.* 2016). Makarova *et al.* (2011) have provided an analysis of the evolutionary relationships between CRISPR-Cas systems and Cas proteins and their classification. After cutting the DNA with the site-directed nucleases (SDNs) the cell can be made to mutate itself with small point changes to the gene (SDN-1) or can provide a template to make larger insertions or deletions to the gene sequence (SDN-2) or provide a template for insertion of a different gene altogether, even from a distant species (SDN-3) (Kuzma *et al.* 2016). The new sequencing technologies addressed two important bottle necks in sequencing, longer sequence lengths and sequence quality. The development of CRISPR/Cas9 has made the process of gene knockout quite easy and it seems to work in most organisms. Using CRISPR/Cas9 system Jiang *et al.* (2013) induced resistance to bacterial blight in rice while Shi *et al.* (2016) developed drought tolerant maize.

   The United States Department of Agriculture (USDA) is not inclined to regulate products developed using CRISPR/Cas9 protocols. Mushrooms developed through this system have not attracted the regulatory oversight as the USDA notes that they do not contain foreign DNA from 'plant pests' such as viruses or bacteria (Waltz 2016).

In addition to the preceding, the following variants of gene editing protocols have also been developed (Wolt *et al.* 2016): (a) homologous recombination (HR), a genetic recombination process where two similar DNA strands exchange nucleotide sequences, (b) non-homologous end joining (NHEJ), a means for repair of double-stranded base sequences without homologous repair, and (c) sight-directed nucleases (SDN), engineered nucleases programmed to specific sites within the genome where they cleave a DNA chain by separating nucleotides, (d) double-stranded break (DSB), causes cleavage in both strands of the DS DNA where the two strands have not separated, and (e) homology directed repair (HDR), a mechanism for DSB repair using DNA sequence homologous to the break site that serves as a template for homologous recombination.

From the ongoing efforts, it looks that it will not be long before GETs deliver fascinating products of nutritional significance.

3.3.6 Moral and Ethical Arguments Against Genetic Engineering Technologies

Actions of individuals, institutions and governments are often judged on the basis of 'moral' and/or 'ethical' criteria and standards, more particularly when the actions are opposed. Though moral and ethical arguments usually come into play in the absence of rational or scientific evidence in support of the opposition as in the case of GE crop technology, there are certainly situations which are rightly qualified as immoral or unethical.

In practise, the terms moral and ethical are used interchangeably, but there is a distinction: morals are an individual's own principles based on religious or cultural beliefs on what is right or wrong, while ethics are rules provided by an external source relating to the right or wrong conduct of groups, institutions and governments.

The development and deployment of GE crops have been castigated as immoral and/or unethical by the activist groups, on the basis of arguments that they, (a) cause harm to human health, (b) damage the environment, (c) have negative impact on traditional farming practises and poor farmers, (d) there is excessive corporate dominance, and (e) they are unnatural, with scientists playing God. Though all these objections have been answered repeatedly on the basis of science and ground results from GE crops commercialised over 20 years in about 30 countries, similar objections will certainly be raised against GE crops with enhanced nutritional qualities, though the traits for nutritional enhancement have no potential to negatively impact human health or the environment, as they are most often based on genes from crop plant species and intended to promote nutrition and health. These traits may also benefit the crops themselves.

Rejecting the activist arguments against GE crops on the basis of moral and ethical grounds, Weale (2010) summarised the position of the Nuffield Council on Bioethics that it is a moral imperative to enable developing countries to take advantage of new technologies, which include micronutrient enrichment. He further stated that, it is a moral imperative to ensure that the farmers in the developing countries are in a position to have access to technologies and to make a choice about their use. Weale (2010) further stated that it is not the task of an ethical analysis to be the champion for a particular technology and that ethical assessment would not affect technological development, especially in cases where the technology addresses urgent human needs. The report on the Study Week of the Pontifical Academy of Sciences on 'Transgenic plants for food security in the context of development' also stated that '*there is a moral imperative to make the benefits of GE technology available on a larger scale to poor and vulnerable populations who want them and on terms that will enable them to raise their standards of living, improve their health and protect the environment*' (Potrykus and Ammann, 2010). Ending food insecurity, hunger and malnutrition is seen as a pressing global ethical priority (von Braun 2010). Mittelstrass (2013) re-emphasised these views and stated that the production of genetically modified food plants has come into the foreground because of its potential to enhance colorific and nutritional quality of crops, in addition to increasing resistance of plants to pests and diseases, as well as improving tolerance to environmental stress and so these technologies should be seen as global public good.

'Do no harm' is the basic global ethic all along. So far none of the GE crops in commercial cultivation have caused any harm and the nutritionally enriched GE crops are far more unlikely to cause any harm.

3.4 Quantitative and Qualitative Modification of Dietary Carbohydrates

3.4.1 The Carbohydrates

Carbohydrates are a very large group of organic compounds ubiquitous in biological systems. They are diverse, simple to very complex molecules, a large number of which are a part of our diet and the major source of energy.

Glucose (dextrose), the primary product of photosynthesis, fructose that occurs in fruits and some vegetables, and galactose which occurs only in bound state, are monosaccharides, the simple sugars. Sucrose, the table sugar from sugar cane or sugar beet, is formed of glucose and fructose while lactose, the sugar in milk is formed of galactose and fructose, are disaccharides. As mono- and disaccharides are small molecules, they can be broken down by the body quickly and are the quickest sources of energy (Beers 2003). The glucans (starch and glycogen), composed of glucose, and fructans (inulin) composed of glucose linked fructose molecules, are polysaccharides, the polymers of thousands of monosaccharides. Because the polysaccharides are large and complex molecules, they take more time to be digested and so release energy slower than the simple carbohydrates (Beers 2003). The liver and muscles store energy as glycogen, a complex glucose containing carbohydrate, which can provide almost a day's worth of calories (Beers 2003). Glucose may form in our body on occasions from other compounds such as TCA cycle intermediates, over a dozen amino acids (glutamic and aspartic acids) and lactic acid, through a process called gluconeogenesis.

There is a large number of carbohydrates involved in plant structures and metabolic functions, but most of them have no direct or significant role in our nutrition. The staple foods such as rice, wheat, corn, potato and so on have the highest concentration of starch and so are its major sources for us. However, some considerable quantity of starch is present in almost all plant foods including those reputed as major sources of protein (legume grains) and there is glycogen in non-vegetarian foods (Table 3.1). Consequently,

Table 3.1 Carbohydrate, protein and fat/oil content of common plant foods.

Food source	Carbohydrates	Proteins	Fats/Oils
Cereals and millets			
Rice	78.0	7.0	1.0
Wheat	71.0	12.0	1.0
Barley	69.0	11.5	1.3
Oats	62.0	12..0	0.7
Corn	66.0	11.0	3.5
Sorghum	72.0	10.0	1.9
Pearl millet	67.0	11.0	5.0
Pulses/Legume grains			
Chickpea	61.0	17.0	5.0
Black gram	59.0	24.0	1.5
Green gram	57.0	24.0	1.3
Red gram	57.0	22.0	1.7
Red Kidney bean	60.0	22.0	1.3
Cow pea	54.0	24.0	1.0
Lentil	59.0	25.0	0.7
Horse gram	57.0	22.0	0.5

Table 3.1 (Continued)

Food source	Carbohydrates	Proteins	Fats/Oils
Peas	56.0	20.0	1.1
Soybean	21.0	43.0	20.0
Ground nut	26.0	25.0	40.0
Tubers			
Potato	23.0	6.0	0.2
Sweetpotato	28.2	1.2	0.3
Tapioca	38.0	0.7	0.2
Carrot	1.6	0.9	0.2
Radish	3.4	0.7	0.1
Beet root	8.8	1.7	0.1
Vegetables			
Cabbage	4.6	1.8	0.1
Cauliflower	4.0	2.6	0.4
Spinach	2.9	2.0	0.7
Tomato	3.6	1.9	0.1
Onion	12.6	1.2	11.1
Pumpkin	4.6	1.5	0.1
Miscellaneous plant food additives			
Apple	14.0	0.5	0.8
Cherry	14.0	1.5	0.6
Coconut	13.5	4.5	42.0
Garlic	29.0	6.5	0.5
Some non-vegetarian foods			
Meat	10.2	22.0	13.3
Crab	3.3	8.9	1.1
Lobster	–	20.5	0.9
Prawn	0.8	19.1	1.0
Liver, sheep	1.3	19.2	1.3
Egg, hen	2.6	13.3	12.8
Milk, cow	4.4	3.3	4.1
Milk, human	7.4	1.1	3.5
Cheese	6.3	24.1	6.3

The macronutrient values given here (in g/100g) are indicative and not absolute. They were taken from diverse sources, ranging from the USDA's National Nutrient Database (USDA 2015) to marketed product package information. They are likely to vary marginally, depending upon a number of factors such as crop variety, maturity, geographical region and so on. Carbohydrates and proteins provide 4 Kcal/g and fats 9 Kcal/g of energy.

there can only be a low-carbohydrate diet but not a no-carbohydrate diet. The issue is the quality rather than the quantity of carbohydrates in foods, also because the general recommendation is to reduce overall carbohydrate consumption (see Section 3.2.3). However, most authorities recommend that about 50 to 55% of the total daily calorie should consist of carbohydrates (Beers 2003).

3.4.2 Modifying Levels of Components of Starch

Starch, our major source of energy, is composed of amylose and amylopectin, two structurally different polymers of glucose. Amylose is generally composed of less than ~3,000 glucose residues with less than 1% of α-1,6 linkages, and more of α-1,4 linkages, while amylopectin is composed of more than ~5,000 glucose residues, with 3-4% of α-1,6 linkages . The enzymes involved in the synthesis of amylose and amylopectin in major food crops have been isolated and it is possible to alter their relative proportions as desired. Questions have been raised on the impact of starch modification on (a) the overall crop yield which is the sum total of starch accumulation, and (b) the food processing qualities of the modified starches such as in wheat used for bread making (Patil and Kameswara Rao 2015).

Most plant starches contain about ~25% amylose and ~75% of amylopectin, with limited intra- and intervarietal variation in their relative proportions. Amylopectin is more rapidly digested in the gastrointestinal tract than amylose, resulting in a rapid release of glucose, subsequently followed by a quick release of insulin, together believed to be an important factor behind the onset of the metabolic syndrome (see Section 3.2.3). Providing healthier dietary starch to the diabetics is the objective of identifying cereal grain varieties (as in rice) with lower glycaemic index and in enhancing amylose and reducing amylopectin. An increased proportion of amylose means enhanced starch that is resistant to digestion and so with a lower glycaemic index, is considered better for diabetics. Starch resistant to digestion is also formed when starch-containing foods (cereals, tubers, legume grains) are cooked and cooled (Yadav *et al.* 2009). Resistant starch is also an important component part of dietary fibre (see Section 3.4.3), that imparts major benefits for colonic health.

The basic conflict of interest is (a) the need for high amylopectin content that yields higher quantities of energy rapidly, for the benefit of the young and others such as athletes, (b) higher quantities of amylose, the digestion resistant starch, that yields limited energy rather slowly, for the benefit of diabetics, and (c) the industrial uses that require various proportions of the two glucans. This has to be achieved without adversely affecting agronomic and commercial interests such as growth and yield. The following crops with altered levels of amylase and amylopectin have been developed:

a) **Rice:** Amylose levels were altered by the expression of a bifunctional and thermostable amylopullulanase in transgenic rice (Chiang *et al.* 2005);
b) **Barley:** The barley mutant '*Himalaya 292*', has a reduced starch content (<18% of dry weight) of which 70% is amylose (Mann *et al.* 2005). The beneficial effects observed in feeding trials with this barley variety on pigs and humans led to the development of a commercial product (BARLEYmax) in Australia;
c) **Wheat:** (i) Development of wheat mutants rich in resistant starch is more difficult as wheat is hexaploid. Nevertheless, a transgenic wheat line with nearly 70% increase

in amylose was developed by suppressing two enzymes (SBEIIa and SBEIIb) by RNAi (Regina *et al.* 2005). When this wheat was fed to rats in a diet as a whole meal, several indices of large-bowel function, including short-chain fatty acids, were improved relative to standard whole meal wheat (Regina *et al.* 2005). These results indicate that high-amylose wheat has a significant potential to improve human health, particularly of the diabetics, through its higher resistant starch content; (ii) By altering levels of key enzymes involved in the regulation of starch synthesis, it is possible to generate starches with new unique properties in the common and durum wheats. Lafiandra *et al.* (2008) have developed a range of mutants with enzymes that control the synthesis of amylose and amylopectin. Crossing of these mutant lines in appropriate combinations resulted in common and durum wheats with novel starches;

d) **Maize:** In maize the amylose-extender mutants have increased proportions of amylose leading to enhanced content of starch resistant to digestion (Liu *et al.* 2009); and
e) **Potato:** (i) Schwall *et al.* (2000) produced a potato with the highest amylose content by simultaneously inhibiting two isoforms of starch branching enzyme (SBE A and B) to below 1%. This starch with very high amylose and very low amylopectin has diverse food and industrial applications; (ii) Amflora (EH92-527-10), a transgenic potato cultivar developed by the BSAF Plant Science to produce over 98% of amylopectin, has several food and industrial applications (Amylogene 2010). Amflora was approved for commercial cultivation in Europe but was withdrawn from the market in the EU in January 2012.

3.4.3 Engineering Levels of Fructans

Fructans are glucose linked fructose polymers that function as anti-freeze in plants. Inulin (not to be confused with insulin) is the commonly occurring fructan in many food plants, more importantly artichoke and garlic, but absent in some like rice. The energy component of fructose is only about a third of that of glucose. For long, only glucose was considered to be the diabetic risk factor which led to encouraging diabetics to consume fructans and high fructose corn syrup, a recommendation not currently favoured. More beneficial effects are that fructans are largely indigestible in the human gut and contribute to dietary fibre (see Section 3.4.3). Along with a few other indigestible carbohydrates, fructans were shown to enhance faecal bifidobacteria which contribute to colon health and reduce the risk of cancer (Bouhnik *et al.* 1999, 2004, Yen *et al.* 2011). The following are examples of crops with genetically enhanced levels of fructans:

a) **Rice:** Normal rice cannot synthesise fructans but Kawakami *et al.* (2008) engineered transgenic rice capable of synthesising fructans;
b) **Potato:** Hellwege *et al.* (1997, 2000) developed a transgenic potato that synthesised the full spectrum of inulin molecules, using genes from artichoke, the standard source of inulin;
c) **Maize:** fructan accumulation through the expression of a bacterial gene was accomplished in transgenic maize (Caimi *et al.* 1996)
d) **Sugar beet:** a high level fructan accumulation was achieved in sugar beet (Sevenier *et al.* 1998)
e) **Chicory:** fructan synthesis enzymes from barley fully expressed in transgenic chicory plants (Sprenger *et al.* 1997).

3.4.4 Quantitative and Qualitative Enhancement Dietary Fibre

Dietary fibre is not just the roughage from over grown vegetables, nor is it all fibre (Patil and Kameswara Rao 2015). It is the tough complex of carbohydrates present in most plant foods (Beers 2003). Some of it is water soluble, digestible and may provide some calories but slows gastrointestinal movement. More of the dietary fibre is indigestible, forms the bulk of faeces and accelerates bowel movement.

There is some variation in the official definition of dietary fibre (Beers 2003; USDA 2005; NIH 2011; Patil and Kameswara Rao 2015). Dietary fibre contains many chemical compounds, mostly derived from the cell walls of plant foods and is formed of (a) starch resistant to digestion and fructans, and (b) non-starch polysaccharides such as cellulose, hemicelluloses and lignin, which are all indigestible. Most common foods contain considerable amounts of both soluble and insoluble fibre.

Although the mechanisms involved in the development of metabolic syndrome (see Section 3.2.3) are not completely understood, increased consumption of fibre-rich foods with low glycaemic index and reduced consumption of highly refined foods, showed a range of significant health benefits, which include reducing the risk of cardiovascular disease, improving glycaemia and glucose sensitivity, assisting weight management, improving bowel health and reducing the risk of certain forms of cancer (Anderson *et al.* 2009), by inducing changes in sugar and cholesterol levels in the blood (Beers 2003) and may even reduce the risk of death from the ailments of the metabolic syndrome (Park *et al.* 2011).

About 25g of fibre is normally consumed, but about 30g is the recommended daily allowance (RDA) (Beers 2003). Since the fibre content of an average serving of fruit/vegetable is only 2 to 4g, enhancement of fibre in the diets is recommended. There are high fibre products on the market which contain additives derived from different biological sources, including bacteria, algae and inedible parts of food plants (Patil and Kameswara Rao 2015). Nestle (2002), while conceding some health benefits from dietary fibre, was critical of the corporate influences that misleadingly promote certain processed foods as the only means of providing adequate dietary fibre.

Diets high in soluble fibre may lead to gas production and constipation, if the water consumed was not adequate. In food products derived from cereals, the health benefits are associated with the consumption of wholegrain products, in which the fibre-rich bran layers are retained. Whole grain wheat flour contains about 10% to 15% dietary fibre compared to 3% in processed wheat. The major forms of dietary fibre in cereals are non-starch polysaccharides derived from the cell wall. In wheat, dietary fibre is principally arabinoxylan (AX) up to 70% and β-glucan up to 20% of the total. β-glucan is of specific interest as soluble β-glucan fractions from barley and oats reduce the risk of coronary heart disease, and were approved by the FDA in the United States. Although the health benefits of cereal food products could be improved by increasing the proportion of wholegrain products, this is difficult to achieve because of lower consumer acceptability, increased cost and difficulties in their processing. Besides, whole grain foods contain more phytate which may affect nutrient availability (see Section 3.10.2). An alternative strategy is to select wheat varieties with higher levels of dietary fibre in their flour, particularly the soluble fibre. Identification of genes encoding for β-glucan synthase in wheat and barley has provided an opportunity to increase β-glucan synthesis in wheat by genetic engineering (Lafiandra *et al.*

2008). Enhancing resistant starch also contributes to dietary fibre content of cereals foods.

3.5 Quantitative and Qualitative Enhancement of Proteins and Amino Acids

3.5.1 The Proteins and Amino Acids

Our body contains large amounts of different kinds of proteins, which are the main building blocks of the organs, tissues and cells. For example, muscle, connective tissues and skin are all built of protein. As proteins are needed in large quantities, they are macronutrients.

Proteins are formed of units called amino acids (AAs) and both proteins and AAs perform diverse important functions in biological systems. About 500 different AAs are known and 23 of them are proteinogenic, the building blocks of (a) peptides which are simple chains of 10 to 50 AAs, and (b) proteins (including most of the enzymes) which are complex molecules formed of hundreds of AAs. The number and sequence of different AAs determine the complex four-level and three-dimensional structure of proteins and their function.

Twenty of the proteinogenic AAs are encoded directly by triplet codons of the genetic code. Cystine, hydroxyproline and hydroxylysine are synthesised from precursor compounds. Some of the proteinigenic AAs are found only occasionally (sarcosine in peanut) or only in animal proteins (thyroxine).

Our body synthesises from the components within the body, many of the AAs we need. But nine AAs, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, cannot be synthesised *de novo* in our body and must come in the diet. These are the essential AAs, the focus of supplementation and/or fortification.

All foods we consume contain a certain quantity of protein (Table 3.1). Some are recognised as protein sources as they contain more protein than the others, such as the legume grains and non-vegetarian foods compared to cereals. Nevertheless, barring a few exceptions, the quantities of carbohydrates and proteins in our major plant foods are about the same (Table 3.1).

We consume proteins for the energy and the AAs, both released on digestion. Adults need to eat about 60g of protein a day (0.8g/kg body weight or 10% to 15% of total calories). Children need more. If more protein is consumed than needed, the body breaks the protein down and stores its components as fat, which can be used for energy released later as needed (Beers 2003). Because proteins are complex molecules, the body takes longer to break them down, and hence proteins are a much slower but longer-lasting source of energy than carbohydrates (Beers, 2003), but both provide only 4Kcal/g of energy.

The proteins in the majority of crop plants are deficient in one or more essential AAs. For example, cereal grains generally lack in lysine and threonine, whereas legumes/pulses are deficient in methionine and cysteine. Consequently, the majority of the world's population that depends on cereal-legume combination diets, suffers from deficiencies of these AAs. About 850 million people worldwide suffer particularly from protein-AA under nutrition (FAO 2004). The WHO considers that protein-energy-malnutrition

(PEM) is the most lethal form of malnutrition and affects every fourth child in the world (WHO 2006). Both the quantity of protein consumed and its quality in terms of the AAs it contains need to be monitored to ameliorate PEM. Among all the essential AAs, lysine and methionine particularly need to be fortified in plant foods as they play several important roles in our metabolism.

Researchers have used diverse breeding techniques, both conventional and modern, to increase the quantity of total protein and the levels of essential AAs, more particularly lysine and methionine, in food crops. In this context, protein biofortification of plant foods through GE crops emerged as a very promising option. Illustrative examples of food crops with enhanced levels or protein and/or selected AAs are detailed hereunder.

3.5.2 Enhancement of Total Protein

The quantity of protein consumed is a factor of awareness of protein-rich foods and affordability. The more important issue is providing properly balanced and adequate quantities of the AAs needed for good health. Nevertheless, there have been some concerted efforts to enhance the total protein content, which also enhances the AA quantities. In this context, the key issue is to ensure that the total amount and composition of storage proteins is not altered to the detriment of the development of the crop plant when attempting to improve AA ratios (Rapp 2002). The following are some examples of enhancement of total protein content:

a) **Maize**: O'Quinn *et al.* (2000) developed a GE maize with higher levels of total protein meant to feed pigs, while Yang *et al.* (2002) succeeded in the expression of a synthetic procine α-lactalbumin gene in the kernels of transgenic maize resulting in higher protein levels. Young *et al.* (2004) prevented the normal loss of the lower floret in a variety of maize to produce kernels composed of a fused endosperm with two viable embryos, resulting in kernels with an increased ratio of embryo to endosperm content, protein and oil, and less carbohydrate (ILSI 2008).
b) **Wheat**: Higher levels of total protein were achieved in wheat by regulating senescence (Uauy *et al.* 2006) and through RNAi (Gil-Humanes *et al.* 2010).
c) **Potato:** The *Ama1* gene from *Amaranthus hypochondriacus*, coding for seed albumin, was expressed in potato, doubling the protein content and enhancing levels of several essential AAs (Chakraborty *et al.* 2000). Li *et al.* (2001) enhanced the total protein content and sulphur-containing AAs in a transgenic potato with 10 ku zein gene from maize.
d) **Soybean:** Increase in the total protein and sulphur containing AAs by over expressing zein protein gene from maize was demonstrated in soybean (Dinkins *et al.* 2001).

3.5.3 Enhancement of Levels of Lysine

Lysine levels were significantly enhanced in the following:

a) In maize free lysine was significantly increased by the transgene dapA (*cordapA*) from the *Corynebacterium glutamicum*, with insensitivity to lysine feedback inhibition (Eggeling *et al.* 1998; O'Quinn *et al.* 2000), which means that lysine levels increase even in lysine-rich varieties.

b) In a climate of neglect of minor cereals and millets in nutritional enhancement efforts, Eswara Reddy and Jacobs (2002) identified five lysine-rich sorghum cultivars which contained 1.5 to 2.0 times more lysine than the others and compared them with a chemically induced high lysine mutant P7210, which was even better than these cultivars. Benmoussa *et al.* (2015) found better seed protein digestibility in an induced high lysine mutant sorghum (P721Q). A transgenic high lysine sorghum was developed a long time ago by Zhao *et al.* (2003), but no efforts were made to process it for commercialisation.
c) The levels of lysine were also enhanced in (i) transgenic rice (Sindhu *et al.* 1997, Katsube *et al.* 1999, Stoger *et al.* 2001, Christou and Twyman 2004); (ii) wheat (Stoger *et al.* 2001; Christou and Twyman 2004); (iii) soybean (Falco *et al.* 1995) and (iv) Canola (Falco *et al.* 1995).

3.5.4 Enhancement of Levels of Methionine

a) Higher levels of methionine, than in the respective isogenics, were achieved in (i) rice (Hagan *et al.* 2003); (ii) maize (Lai and Messing 2002); and (iii) chickpea (Chiaiese *et al.* 2004). In the tubers of an antisense potato (cv. Désirée), methionine level increased by a factor of 30, without a simultaneous reduction in threonine, unlike in its leaves (Zeh *et al.* 2001).
b) Methionine-rich 2S albumin gene from the Brazil nut (*Betholletia excelsa*) was introduced into transgenic soybean (Saalbach *et al.* 1994), to improve its nutritional quality. Brazil nut is known to be allergenic in a few individuals (Nordlee *et al.* 1996) and the transgenic soybean would probably have been allergenic only in such individuals. Nevertheless, activists have come out heavily against this product leading to its withdrawal from further processing.

3.5.5 Simultaneous Enhancement of levels Several Amino Acids

The following are the successful attempts to enhance several AAs in the same variety:

a) **Maize:** LY038, the '*opaque-2*' maize developed through gene silencing (RNAi) (Segal *et al.* 2003), is low in zein seed storage proteins, with a compensatory increase in lysine and tryptophan in non-zein seed proteins as well as free lysine and tryptophan, compared to the isogenic (Dizigan *et al.* 2007). Lysine was synthesised in *opaque 2* by a branch of a pathway which also leads to the synthesis of three other essential AAs, threonine, methionine and isoleucine (Galili 2002). The LY038 maize was successfully introduced for cultivation in a number of countries (Lucas *et al.* 2007).
b) **Potato:** Li *et al.* (2001) have enhanced the levels of the sulphur containing AAs in transgenic potato with the 10 ku zein gene from maize.

3.5.6 Artificial Storage Protein

An interesting attempt was made to boost the levels of selected AA to match the human AA requirements, deploying a completely synthetic gene coding for an artificial storage protein (*asp-1*) containing an optimum number of essential AAs (methionine, threonine, lysine, and leucine), which was incorporated into sweetpotato (Egnin and Prakash 1997, Prakash and Jaynes 2000). Besides a significant increase in the levels of essential AAs, this also resulted in a two- to five-fold increase in total protein that resists degradation by proteases. The same gene successfully expressed also in cassava (Zhang *et al.* 2003). The expression of a synthetic procine α-lactalbumin gene in maize resulting in higher protein (Yang *et al.* 2002), was already discussed.

3.5.7 Alternate Interventions

The technological interventions used to produce diverse protein-AA enriched crops detailed in the preceding sections are extremely precise and efficient. Certain contingent issues like the enormous time and expense needed for their development and the regulatory and political hurdles preventing their commercialisation, make it necessary to adopt alternate interventions to ameliorate PEM, till the genetically biofortified crops are marketed. The following are some suggestions in this direction, but as the benefits from these foods are not common knowledge public education would be the crucial issue.

a) **Choosing food combinations rich in lysine/methionine:** The RDA for lysine is 30mg/kg/day. Soybean has about the same amount of lysine as egg (5.3 g /100 g). While there are a number of meat, fish and dairy products with high amounts of lysine (2-3 g /100 g), lysine-rich plant foods are hard to find. Among these, peas, fenugreek, different beans, cowpea and sesame seed contain about 1.5-1.8 g/100 g of lysine (UAB 2011).

   The RDA for methionine is 10.5mg/kg/day. Non-vegetarian foods contain appreciable quantities of methionine but vegetarian foods (including soybean) are particularly poor in this AA. Again it is egg that has high methionine content among all foods (2.7 g/100 g) with dairy products containing far less (0.8 g/100 g). Sesame seeds (1.65 g/100 g) and tree nuts (1.0 g/100 g) are probably the richest in methionine among plant foods while some methionine is obtainable from poppy seed and sunflower seed (0.4 g/100 g).

b) **'Complete' proteins:** In comparison with proteins from non-vegetarian and dairy foods, plant foods have a relatively low biological protein value in terms of qualitative and quantitative content of particularly the essential AAs. Nevertheless, they contain at least trace amounts of all of the AAs that are needed in human nutrition and so are 'complete' (McDougall 2002) or nearly complete proteins. Certain traditional combinations of foods containing cereal grains (wheat, corn, rice) and legumes (different beans, peanuts), such as corn and beans, rice and soybean or rice and red Cajun bean, and others seem to contain adequate amounts of the essential AAs (UAB 2011). The Indian practise of consuming chickpea or pigeon pea with products of wheat or rice belongs to this group.

Woolfe *et al.* (2011) developed a computational quantitative tool called 'vProtein', to identify optimal AA complements (particularly the essential AAs) in plant-based foods, and to identify various combinations of plant foods to provide proteins of higher biological value, so that all the AAs needed are provided in adequate quantities. Wheat germ, cauliflower, garlic, cinnamon and tomatoes are among the top 10 single plant foods with the most balanced content of essential AAs (Woolfe *et al.* 2011). vProtein analyses suggest that pairings of plant-based foods should be based on the individual foods themselves instead of on broader food group-food group pairings. The most efficient pairings include (a) sweet corn/tomatoes, (b) sweet corn/cherry, and (c) apple/coconut (Woolfe *et al.* 2011). The macronutrient content of some of these foods is indicated in Table 3.1. The top pairings also highlight the utility of less common protein sources such as the edible seaweeds, *Spirulina*, and leaves of pumpkin and drum stick tree. From a public health perspective, many of the food pairings by vProtein represent novel, low cost food sources to combat malnutrition. Interestingly, the vProtein study found no statistically significant bias towards grain/legume pairings for protein complementation suggested by earlier studies (McDougall 2002, UAB 2011).

3.5.8 Non-Proteinogenic Amino Acids

All the while research on AAs has been focused on the proteinogenic AAs. There are over 400 non-proteinogenic AAs occurring freely in many edible plants (Mamatha Rao 2008) and their impact on our health is hardly known. Some of them should certainly prove to be beneficial. As at present three non-proteinogenic AAs, carnitine, creatine and γ-aminobutyric acid (GABA), seem to be worthy of attention. Their supplements are already on the market, but providing them through food is certainly better and safer and this needs concerted research efforts.

a) **γ-AminobutyricAcid:** γ-aminobutyric acid (GABA) occurs freely in many edible plants such as rice, potato, tomato, mustard and so on. GABA rapidly accumulates in plant tissues in response to biotic and abiotic stress. It regulates plant growth (Ramesh *et al.* 2015) and is a signalling molecule in plants in diverse situations (Roberts 2007). In humans, GABA is synthesised in the brain from glutamate and directly regulates muscle tone. When orally administered, GABA increased the quantity of human growth hormone (Powers *et al.* 2008). More interestingly, GABA lowers hypertension (blood pressure) (Kowaka *et al.* 2015). A GABA-fortified transgenic Joponica variety (cv. Koshihikari) of rice contains 3.5g/kg of GABA, while the isogenic has 0.8g/kg. A field trial of a diet containing 2.5% of GABA-fortified rice for eight weeks had significantly(20mm/HG) reduced blood pressure in spontaneously hypertensive rats, indicating that GABA-fortified foods could control or even prevent hypertension (Kowaka 2015).
b) **Carnitine:** Carnitine is essential in mobilising fat from cytosol to the mitochondria facilitating energy release (Steiber *et al.* 2004, Karanth and Jeevarathnam 2010, Ehrlich 2011). Lysine and methionine are needed for the synthesis of carnitine. The proper utilisation of fats and oils, which release 9Kcal/g of energy, is important not only for the energy budget, but also to reduce their accumulation in the body, all of which are facilitated by carnitine.
c) **Creatine:** Creatine (often confused with creatinine, the indicator of kidney function), is synthesised in the liver from glycine and arginine, and stored mostly in skeletal muscles. Creatine is a readily available source of energy in the body (Beers 2003). In the diet it comes from milk, red meat and some fish. Some studies indicate that creatine increases work performance and its deficiency particularly affects vegetarian children and may cause severe neurological defects (Braissant *et al.* 2011), warranting its supplementation and/or fortification in common plant foods.

3.6 Quantitative and Qualitative Enhancement of Fatty Acids in Oil Seed Crops

3.6.1 Lipids, Fats and Oils

The terms lipids, fats and oils are often interchangeably used for the edible fats and oils (EFOs), from either plant or animal sources. The term 'lipids' is more inclusive of edible fats, oils and waxes, as well as fat-soluble compounds such as sterols, vitamins, cholesterol, glycerides, lipoproteins, glycolipids, phospholipids and so on.

Fats are semisolid and oils are liquid at the body temperature. All foods we consume contain a certain quantity of different kinds of lipids (Table 3.1), while those EFOs used in cooking food or those added to it in quantities constitute the direct sources. The chemistry, synthetic pathways and biological roles of the EFOs are complex. Fundamentally the EFOs are composed of fatty acids (FAs) formed of unbranched carbon chains. The FAs occur in our body system as triacylglycerols (triglycerides), units of three (same or different) FAs associated with a glycerol molecule. The triglycerides are the major storage form of energy and need to be broken down (digested) by lipases, so that the FAs are released and absorbed in the blood stream to be used by the body system. The quantity of the EFOs we consume and the FAs they contain, as well as the quantity of cholesterol in our blood are regarded as crucial in maintaining health.

3.6.2 Cholesterol

Strictly speaking, cholesterol is a steroidal compound and is not fat as it is not formed of FAs. Cholesterol is treated along with fats as it is soluble in fat solvents and is associated with fats in our body. No plant food contributes cholesterol to our body. Cholesterol is essential for the formation of cell membranes and synthesis of bile acids. Cholesterol is a precursor of many steroidal compounds in mammalian metabolism, particularly sex hormones. Cholesterol may have a negative impact on our health when it is a component of 'plaque', which narrows down or even clogs blood vessels. Plaque is composed of 'fatty' material containing cholesterol, platelets (small blood cells), collagen and calcium. Cholesterol also complexes with lipids and proteins, to form 'low-density lipoproteins' (LDL) and 'high density lipoproteins' (HDL), both with significant implications in cardiovascular disease.

3.6.3 Characterisation of Fatty Acids, Dietary Fats and Oils

The FAs and EFOs are characterised on the basis of diverse criteria:

i) Basing on the number of carbon atoms in the carbon chain, a FA is short if it is formed of less than six carbon atoms (butyric acid), medium if it has 8 to 14 carbon atoms (lauric, myristic acids), long if the number of carbon atoms is between 15 and 21 (palmitic, oleic, stearic acids) and very long when the chain is formed of 22 or more carbon atoms (erucic, cerotic, lignoceric acids).
ii) Basing on the number of carbon-carbon double bonds in the carbon chain, a FA is saturated (SFA) if without any double bonds (palmitic, stearic acids), monounsaturated (MUFA) if with one double bond (oleic, euric acids) and polyunsaturated (PUFA) if with more than one double bond (linoleic, α-linolenic, eicosapentaenoic (EP) acids).

iii) Basing on the position of the double bond on the carbon chain counted from its free end, some FAs are omega-3 FAs (α-linolenic, EP acids), omega-6 FAs (linoleic, arachidonic (AR) acids) or omega-9 FAs (oleic and erucic acids).
iv) The FAs which the mammalian system cannot synthesise and so must come from the diet are the Essential FAs. They include the omega-3 FAs (α-linolenic, EP, and docosahexaenoic (DH) acids), and omega-6 FAs (linoleic and AR acids). The EPA and DHA are the long chain PUFAs (LC-PUFA), abundant in fish oils, are essential for brain development and vision. The possible protective roles in arthritis, hypertension and heart disease, attributed to these FAs, have enormously increased their popularity (Chrispeels and Sadava 2003). Linoleic and α-linolenic acids are found in vegetable oils. In our body system, ARA can be formed from linoleic acid, while EPA and DHA can be formed from α-linolenic acid, which makes linoleic and α-linolenic acids the really essential FAs (Beers 2003), although stearidonic acid, an omega-3 FA, was found to be more efficient than α-linolenic acid as a precursor of EPA and DHA (James 2003).
v) *Trans*-fatty acids are saturated by new hydrogen atoms attached at a side/site on the carbon chain that is geometrically opposite to the normal *cis*-position. There are naturally occurring *trans*-FAs such as the elaidic and vaceenic acids found in milk products. *Trans*-FAs are mostly induced through hydrogenation of vegetable oils that converts unsaturated FAs into SFAs making them and the EFOs such as margarine containing them more solid and stable preventing rapid rancidification. *Trans*-FAs are not formed during cooking but form when *cis*-polyunsaturated FAs pass through rumen of livestock due to gut bacterial action and so are present in the dairy products. Consumption of EFOs containing large quantities of *trans*-FAs was discouraged for decades as they may increase cholesterol levels in the body, which may contribute to the risk of atherosclerosis. Many countries have banned *trans*-FAs in foods. Nevertheless, higher concentration of plasma *trans*-palmitoleic acid in dairy foods is now associated with higher concentrations of HDL and lower concentrations of triglycerides, which together lower the risk of diabetes in adults (Malhotra 2013).
vi) The EFOs are composed of different FAs in varying proportions and are grouped as saturated, monounsaturated or polyunsaturated, depending upon the major kind of FAs contained in each. Accordingly, butter and coconut oil containing 56% and 90% saturated FAs respectively, are saturated EFOs. Groundnut oil (46%); sunflower oil (83.5%) olive oil (70%) and rice bran oil (38%) are monounsaturated EFOs while sesame oil (41.7%) and soybean oil (57.7%) are polyunsaturated EFOs. The EFOs containing a higher proportion of saturated FAs are more semisolid than liquid. The relative proportions of SFA, MUFA and PUFA can be genetically altered as detailed hereunder.

3.6.4 Quantitative and Qualitative Improvement of Oil Seed Crops

The EFOs have become an important target largely for quality improvement in terms of the FAs they contain because of many crucial roles different FAs play in our metabolism such as control of inflammatory responses, blood pressure regulation and cardiovascular health (Beers 2003, Newell-McGloughlin 2010, Patil and Kameswara Rao 2015). The EFOs are a major source of our energy as they provide nine Kcal/g, double that from carbohydrates or proteins. Many EFOs are good sources of vitamin E (wheatgerm, sunflower, safflower, cotton seed oils) and some contain efficient thermostable antioxidants such as oryzanol in rice bran oil (see Section 3.9). EFOs are essentially needed for the absorption and utilisation of fat-soluble vitamins A and D (see Section 3.7) and antioxidants like lycopene (see Section 3.9).

Genetic modification of oilseed crops can provide an abundant and relatively inexpensive source of dietary FAs (Reddy and Thomas 1996, Kinney and Knowlton 1998, Liu *et al.* 2002a,b, Wallis *et al.* 2002, Anai *et al.* 2003, Abbadi *et al.* 2004, Newell-McGloughlin 2010) and the production of appropriate kinds and quantities of the fatty acids in plant foods obviates the need for major dietary modifications (Damude and Kinney 2008, Newell-McGloughlin 2008). Impressive progress has been made with transgenic approaches for the modification of oil content in plants (Newell-McGloughlin 2010). Novel pathways that are normally absent from plants, for an enhanced expression of LC-PUFAs have been reconstructed from bacterial pathways (Tucker 2003, Damude and Kinney 2008). While most of the research was conducted using transgenic technology, some used gene silencing (Liu *et al.* 2002a, 2002b) or TALEN based mutations (see Section 3.3.5) (Huan *et al.* 2014) to design EFOs with novel and desirable combinations and levels of fatty acids. The following are illustrative examples of EFOs genetically altered for better quantities and combinations of FAs:

i) **Maize:** Total oil content in maize kernels was enhanced along with protein levels and simultaneously reducing the carbohydrate (Young *et al.* 2004, ILSI 2008). Though it is the quality of the oil in terms of its FAs that is important, technologies to enhance the total seed oil content too would be useful to raise the levels of chosen FAs.
ii) ***Arabidopsis*:** Qi *et al.* (2004) deployed three genes that express in the seeds of *Arabidopsis thaliana* to obtain higher levels of EPA (3%) and ARA (6.6%) in the vegetative tissues without affecting plant growth and development. Robert *et al.* (2005) succeeded in obtaining higher levels of DHA, up to 0.5% of the total FAs, in *Arabidopsis.* While *Arabidopsis* is not a source of DFOs, these results are important in that the modified biosynthetic pathways can be incorporated into other oil plant systems, and to induce synthesis of large quantities of extractable EFOs in the vegetative tissues.
iii) **Prevention of formation of *Trans*-FAs:** The formation of *trans*-FAs in the rumen of livestock from *cis*-polyunsaturated FAs is prevented by polyoleosins produced by genes from sesame introduced into forage grasses to encapsulate triglycerides within self-assembling micelles, which protects them from bacterial activity during passage through the rumen (O'Neill 2007).
iv) **Rapeseed (*Brassica rapa*) oil**: Rapeseed oil, an industrial lubricant and a source of biodiesel in the temperate countries, was not suitable for human consumption, on account of its sharp taste due to glucosinolates (500μmol/g). Besides, it contains over 60% erucic acid linked to several ailments including cancer. In 1974, the Canadian plant breeders developed the canola crop (*Can*adian *O*il *L*ow *A*cid) from the oil seed rape, genetically modified by conventional breeding techniques, to make it fit for human consumption. Canola, a great scientific and commercial success, has less than 2% of erucic acid and less than 30μmol/g of glucosinolates. It is projected as a healthier edible oil as it contains about 70% of MUFAs such as oleic acid, more than most other edible oils. Canola seed cake can be used as cattle and poultry feed.

Development of canola is a great achievement in altering the chemistry of an edible oil with enhanced health benefits. During the past two decades, GE varieties of canola for resistance against pests, diseases, drought and herbicides were developed to promote its wider cultivation and today 98% of all commercially cultivated canola is GE for herbicide tolerance.

Several other developments make canola an important and improved edible oil. The more significant among them are (a) a GE canola variety with lauric acid totally replacing oleic acid (Del Vecchio 1996), makes the oil more resistant to rancidification, and reduces the need for hydrogenation which leads to the formation of the unhealthy *trans*-FAs; (b) producing omega-3 FAs in canola using a gene from the fungus *Mortierella alpina* (Ursin *et al.* 2000, 2003); (c) a transgenic canola with 23% stearidonic acid, a more efficient precursor for the synthesis of the other omega-3 FAs (James *et al.* 2003); (d) maximising the accumulation of Omega-3 LC-PUFAs (Kinney *et al.* 2004); and (e) successful modification of FA composition through a reconstitution of the biosynthetic pathways (Damude and Kinney 2008) to develop a GE canola containing high levels of stearic and linolenic acids.

v) **Mustard (*Brassica juncea*) oil**: Wu *et al.* (2005) developed a GE mustard with a significant increase in the levels of ARA and EPA, though the accumulation of DHA was low.

vi) **Soybean oil**: (a) a cyanobacterial delta 6-desaturase gene resulted in the production of γ-linolenic acid in transgenic soybean (Reddy and Thomas 1996); (b) gene-silencing enhanced oleic acid content to 80% level as against normal 23% in soybean (Kinney and Kowlton 1998); (c) genes from two fungi, *Mortierella alpina* and *Saprolegnia diclina*, resulted in a 3% level of DHA in soybean (Kinney *et al.* 2004); and (d) four soybean lines with very low levels of PUFAs were developed by introducing mutations in two FA desaturase 2 genes (*FAD2-1A* and *FAD2-1B*) using TALEN based protocols (Huan *et al.* 2014). The FA profile was dramatically changed in plants homozygous for mutations in both the genes. Oleic acid increased from 20% to 80% and linoleic acid decreased from 50% to less than 4%. The high MUFA containing products developed by Kinney and Kowlton (1998) and Huan *et al.* (2014) do not need hydrogenation which eliminates the development of *trans*-FAs.

vii) **Palm oil:** The oleic and stearic acid content of palm oil was enhanced and that of palmitic acid lowered (Jalani *et al.* 1997, Parveez 2003).

viii) **Cotton seed oil:** Chapman *et al.* (2001) developed a transgenic cotton variety with high oleic acid. Liu *et al.* (2002a, 2002b) used hairpin RNA-mediated gene silencing to down-regulate the expression of the genes *ghSAD-1* and *ghFAD2-1* in the cotton seed, which increased stearic acid levels from 2-3% up to 40%. Silencing the *ghFAD2-1* gene has greatly elevated oleic acid content from 15% up to 77%, simultaneously reducing palmitic acid levels. Stacking two genes did not result in any diminution in the degree of silencing.

ix) **Rice seed oil:** Anai *et al.* (2003) introduced the soybean microsomal omega-3 fatty acid desaturase gene to enhance the levels of α-linolenic acid in the rice seed oil.

x) **Linseed oil:** Linseed oil is being promoted as the sole vegetarian source of omega-3 FAs. Abbadi *et al.* (2004) have elucidated the biosynthesis of VLC-PUFAs in transgenic oil seed systems and enhanced the levels of omega-3 (EPA) and omega-6 (ARA) FAs in linseed oil.

xi) **Safflower seed oil:** While different varieties of safflower with major content of either MUFA or PUFA are on the market, in one the γ-linoleic acid was specifically enhanced (Arcadia Biosciences 2008).

3.6.5 The New Shift in Fat Paradigm and Its Implications

The widely publicised concepts of the Framingham study, American Heart Association, American Cancer Society, American Society for Nutritional Sciences, U.S. National Institutes of Health, World Health Organisation and European health organisations have influenced public opinion in the west to adopt the food and nutritional advice, particularly regarding the intake of EFOs and FAs. A number of beliefs on good and bad EFOs and cholesterol, which were also promoted by commercial interests, have greatly influenced the public mind. Most of these long-standing beliefs have been questioned from time to time, but without much response. In recent times Malhotra (2013) and CSRI (2015) strongly raised the issues that need a reassessment of evidence and modification of advice proffered to the public. Some of the important issues are:

a) Over the last 50 years, general nutritional wisdom has recommended moderate consumption of fat, lower intake of saturated fats (butter, lard, milk, red meat, coconut oil) and cholesterol (eggs, poultry, beef) and increased consumption of polyunsaturated fats and carbohydrates (CSRI 2015). Some believe that these dietary recommendations are actually the leading cause of high obesity levels and the fast growing number of people suffering from metabolic syndrome (see Section 3.2.3). The higher intake of vegetable oils and the increase in carbohydrate consumption in the last 30-40 years are the two leading factors behind the high rates of obesity and metabolic syndrome in the United States, and not saturated and monounsaturated fats (CSRI 2015).
b) The link between saturated fat intake and cardiovascular risk was never been proven (CSRI 2015). The advice to avoid saturated fats derived from dairy foods was incorrect as these foods provide vitamins A, B_{12} and D, whose deficiency increases the risk of cardiovascular disease and calcium and phosphorus in dairy foods are antihypertensive (Malhotra 2013).
c) Now butter is considered better than margarine which is high in *trans*-fats, but not all *trans*-fats are harmful, as for example *trans*-palmitoleic acid (Malhotra 2013).
d) Red meat which is high in saturated fats is safer than processed meats which contain higher levels of added sugars, nitrates and sodium (Malhotra 2013).
e) There is no unanimity on the intake of either EFOs or different kinds of FAs. No RDA was proposed for omega-3 FAs but 200mg/day is believed to benefit cardiovascular health, while the total EFO intake from direct sources should be limited to less that 30% of total calorie consumption (about 90g/day). Some experts recommend only 10g/day (Beers 2003), but low fat diet results in an unhealthy lipid pattern and increased insulin resistance, compared to many carbohydrate diets (Malhotra 2013).
f) The quantity of cholesterol eaten has basically no influence on the level of cholesterol in the blood (CSRI 2015). The belief that high cholesterol alone is a risk factor in cardiovascular disease is inaccurate. Free cholesterol is often clinically reduced by using the synthetic 'statins', in spite of several unacceptable side effects (Zhang *et al.* 2013). Serum cholesterol can be controlled by dietary saponins in such foods as oats and tomato (Kameswara Rao 2000). On the other hand, even low cholesterol levels are associated with mortality from stroke, heart disease, and cancer (Nago *et al.* 2011).
g) The ratio of omega-3 and other omega FAs is crucial rather than the quantity of intake of any of them (Simopoulos 2003). There is little or no evidence for the

effectiveness of treating any health conditions using either γ-linolenic acid (GLA), an omega-6 PUFA in vegetable oils, or its rich supplements (ACS 2013).

h) The important conclusions from the CSRI fat document (2015) are (a) natural unprocessed fats and natural foods high in monounsaturated and saturated fats are one of the preferred sources of energy for our bodies to use and store, and (b) omega-3 FAs have strong protective properties for our heart and brain.

Exploiting prevailing beliefs, commercial interests projected some EFOs and FAs as better than others for our health, dissuading the public from using most of the traditional products and dietary practises. Research on the quantitative and qualitative improvement of EFOs and FAs also was based on long-standing beliefs, many of which are now being questioned. Most of the existing biofortified products detailed in this chapter were developed using the expensive and time-consuming GE protocols and except for canola none are being commercialised. As per the new fat paradigm (Malhota 2013, CSRI 2015) most of them cannot be recommended for public consumption and so have no prospects of commercialisation. Since the observations and conclusions of Malhothra (2013) and CSRI (2015) are critical in deciding the quality and quantity of intake of EFOs and FAs, it is better to re-examine the evidence before we choose any EFO crop for improvement.

3.7 Enhancement of Levels of Vitamins

3.7.1 The Vitamins

Vitamins play crucial roles in our metabolism. There are about 20 vitamins that constitute a vital part of a healthy diet. In addition to the roles of the individual vitamins, they in turn affect many other metabolic processes that make the difference between health and disease. Some vitamins are needed for the synthesis of some others, as for example, folate (vitamin B_9) for the synthesis of pantothenic acid (vitamin B_5).

Vitamins are essential micronutrients, needed in small amounts. The human body can synthesise only the retinol and derivatives (vitamins A_1 and A_2), ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), if their precursors come through diet. Some like cobalmin (vitamin B_{12}) come exclusively from animal products such as meats, eggs and milk. Vitamins A, D and B_{12} are stored in the liver in small quantities and the other vitamins cannot be stored in the body. Hence, all vitamins need to be continuously supplied in diet, which makes them essential nutrients.

There is a certain amount of confusion regarding the nomenclature of vitamins on account of (a) the chemical names changed often with better understanding of their biochemistry and (b) the usage of the chemical names *vs* the convenient but not so precise popular alphabet-based names.

Vitamins A, D, E (tocopherols) and K (quinines) are fat-soluble, need fats for absorption and function, accumulate in the body and excess intakes are undesirable. Vitamins of the B group, such as thiamin (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9), cobalamin (B_{12}), and vitamin C (ascorbic acid) are water soluble, excreted in the urine, and can rarely reach toxic levels.

The Recommended Dietary Allowances (RDAs), which are the amounts of each vitamin most people need each day to remain healthy, and the limits of safe consumption have been determined for most vitamins (Nestle 2002, Beers 2003), but there are no convenient tests to determine the levels of different vitamins in the body. The RDAs are suggestive at the population levels and not necessarily for individuals, which vary basing on a number of factors such as diet, age and general health. The metabolic roles, effects of both deficiencies which arise out of low intake or impaired fat absorption, and excess intake of vitamins, are well recorded (Beers 2003).

Consuming too little or too much of certain vitamins can cause nutritional disorders. For example, an excess of folate (B_9) may mask a deficiency of B_{12} (cobalamin) (Nestle 2002).

People who eat diverse diets containing legume grains (pulses), fruits and vegetables are unlikely to suffer from vitamin deficiencies. Legume grains are a good source of several vitamins. Germinated gram seeds contain increased amounts of pantothenic acid, biotin, niacin, riboflavin, thiamine, cobalamine, ascorbic acid and tocopherol compared to dry seeds (Mamatha Rao 2008).

Large sections of populations in many countries, particularly the emerging economies, seem to be deficient in vitamins A, B_9, B_{12}, C, D, E and K. These vitamins are usually targeted for supplementation through tablets, capsules and fortified foods and drinks. There has been much hyped information on the benefits of these vitamin supplements (Nestle 2002), resulting in consumption of megadoses without medical supervision, which may lead to harmful effects (Beers 2003).

There have been some impressive products of biofortification of some widely consumed food crops with the more crucial vitamins or their precursors, as detailed here.

3.7.2 Retinoids (Vitamin A)

The retinoids (retinal, retinol and its esters, and retinoic acid) are a group of carotenoid derivatives, which denote Vitamin A. Retinol is a component of photoreceptive nerve cells in the retina and is essential to prevent night blindness and to maintain good eyesight. The other forms of retinoids have different functions such as keeping the skin and epithelial linings of the internal organs healthy as well as in maintaining immunity against diseases.

Vitamin A is abundant in fish oils, liver, egg, butter, cheese and cream. Dairy and meat products primarily contain retinyl esters (largely with palmitic acid), which are converted into retinol in the small intestine, and subsequently to retinal or retinoic acid. A small quantity of vitamin A is stored in the liver.

Mammals, including humans, cannot synthesise retinoids *de novo* but can if the precursors α- and β-carotenes (often inaccurately referred to as pro-vitamin A), are available in the body. Among these two carotenoids which can come only from plant foods, β-carotene is more important as it is readily converted into retinoic acid. Two other carotenoids, α-carotene and β-cryptoxanthin, are intermediates rapidly converted into downstream products (Farre *et al.* 2010, Zhou *et al.* 2011). Green leafy vegetables, yellow vegetables and fruits, and red and brown sea weeds are rich in carotenoids (including α- and β-carotenes), all of which also function as antioxidants (see Section 3.9).

The RDA of vitamin A is 700-900-1,300μg, depending upon the age group, sex and pregnancy, with an upper limit of 3,000μg (Beers 2003). A syndrome of disorders, collectively called vitamin A deficiency (VAD) disorders, sets in if vitamin A levels are inadequate for longer periods. Rice plants produce β-carotene in the green tissues but not in the edible grain, even though the biochemical machinery needed for its synthesis is all present in it. Dependence on cereals such as rice, with poor or no vitamin A precursor content, as the predominant food source can lead to VAD disorders.

VAD is widely prevalent in the developing world affecting nearly half-a-million children every year. It is the major cause for blindness. Annual mortality rate from VAD is closer to 3 million. In China, 60% of the rural and 30% of the urban population suffer from VAD (Dubock 2014).

Pregnant women have a higher demand for vitamin A, and currently more than 20 million pregnant women in developing countries suffer from VAD and nearly 6 million of these women suffer from night blindness, with half of the cases occurring in India (Patil and Kameswara Rao 2015).

Vitamin A is supplemented in tablets, capsules, fortified drinks, which benefitted mostly the urban and educated and not the poor in the developing countries. Foods with the algae Spirulina (*Arthrospira platensis)* and *Dunaliella marina* are also widely used for vitamin A precursor supplementation. Since 1990, vitamin A capsules have been provided to many at-risk populations at costs of US$ 1 billion a year, which has certainly been helpful in saving millions of lives. But this does not address the underlying vitamin A status of the targeted populations nor is sustainable because of heavy recurring costs. A more functional approach is to enrich the common food crops with the vitamin A precursor carotenoids, particularly β-carotene, so that the vitamin A precursors become available on consumption, without an additional effort and/or expense and so food fortification should take over (Dubock 2014). The successful introduction of a conventionally bred orange-fleshed sweetpotato rich in β-carotene in Mozambique and Uganda (Evans *et al*. 2014) is a welcome effort in this direction. Too often there are unsubstantiated claims of carotenoid (or other nutrient) rich conventional or conventionally bred foods used in arguments against modern technological intervention. There is a pressing need to go for modern technological interventions to enrich the staple crops, rather than secondary food crops.

The biosynthetic pathways of α- and β-carotenes are now so well understood that their levels are significantly enhanced not only through rDNA technology, but also by silencing the competing genes to redirect the flux (Diretto *et al*. 2007a). Procedures were devised to introduce the metabolic pathways into widely consumed plant foods to facilitate β-carotene synthesis in their edible parts. High β-carotene levels were achieved in a number of food crops such as (a) rice (Ye *et al*. 2000, Paine *et al*. 2005), (b) maize (Aluru *et al*. 2008, Naqvi *et al*. 2009; Pixley *et al*. 2013), (c) sorghum (Wambugu *et al*. 2012, Anonymous 2014), (d) potato (Ducreux *et al*. 2005, Lu *et al*. 2006, Diretto *et al*. 2007a,b, 2010, Zhou *et al*. 2011), (e) tomato (Romer *et al*. 2000, Rosati *et al*. 2000, Fraser *et al*. 2001, 2002, Dharmapuri *et al*. 2002), (f), cassava (Welsch *et al*. 2010), (g) sweetpotato (Evans *et al*. 2014), (h) canola (Shewmaker *et al*. 1999, Ravanello *et al*. 2003), and (i) flaxseed (Fujisawa *et al*. 2008).

3.7.2.1 The Golden Crops

β-carotene imparts a golden-yellow-orange colour to tissues in which it is present, the intensity of the colour being relative to its concentration. Alluding to the colour, crops enriched with β-carotene are enthusiastically and optimistically called 'Golden Crops'. Three of these, the golden rice, golden sorghum and orange maize have gone much far beyond the proof-of-the-concept stage. A multivitamin corn holds a great promise. While the enriched sorghum and maize crops would immensely help the people in

South America and Africa, golden rice is essentially needed by the rice eating people in the Asian countries.

3.7.2.2 Golden Rice

The first and the most promising among the golden crops is the β-carotene enriched rice named golden rice (GR) (Ye *et al.* 2000, Paine *et al.* 2005), which has raised, a decade and half ago, yet unrealised hopes of ameliorating VAD disorders in the developing countries.

Ye *et al.* (2000) initially developed GR using three genes, two from the garden daffodil and one from *Pantoea ananatis* (syn. *Erwinia uredovora*), to reconstitute the entire pathway enabling the accumulation of 2μg/g of β-carotene in the endosperm. Almost immediately, the activists condemned GR1, on various counts. In addition to the usual anti-GE crop charge sheet, the more specific objections, none scientifically established, raised against GR1 were, (a) the quantity of β-carotene is insufficient to have a significant impact on human nutrition, a charge also supported by Nestle (2001), (b) the phytoene synthase gene was taken from daffodil which contains a large number of toxic chemicals and so not safe for human consumption, and (c) GR1 contains hygromycin resistant marker genes which on lateral transfer make hygromycin based human treatment ineffective (Kameswara Rao 2015). Nevertheless, responding to these concerns, an improved version called GR2 was developed, in which, (a) the hygromycin resistant marker was replaced by mannose phosphate isomerase, (b) a more efficient homologue of the phytoene synthase gene was taken from maize and (c) the concentration of β-carotene was raised to 37μg/g (Paine *et al.* 2005).

The GR2 event has been successfully transferred into a large number of selected locally adapted rice varieties in the Philippines, Bangladesh, Vietnam, India and China, by the International Rice Research Institute (IRRI) in Philippines, and the respective public sector national research establishments in these countries. It is expected that GR2 event-incorporated rice lines will provide the RDA of β-carotene in 100-200g of rice, which corresponds to the daily consumption by children.

A very large number of tests were conducted on GR1 and GR2 on a wide range of parameters of efficacy and safety for over a decade and half, in different countries, in the laboratory, green house and open fields. The data were very encouraging, building up the hope of regulatory approvals for commercialisation of GR2. Then, an unfortunate event in China has placed an additional burden on the prospects of commercialising GR2. This relates to feeding tests involving many children in the Hunan Province conducted in 2008 jointly by the Chinese scientists and a team from the Tufts University in the United States. These tests were actually designed to answer the activist charge that the efficacy of β-carotene was never clinically proved in human trials, in spite of the fact these cannot be conducted until a degree of human safety was demonstrated. Tang *et al.* (2009, 2012) who have conducted these trials concluded that the β-carotene in GR2 is as effective as pure β-carotene in oil and better than that in spinach at providing vitamin A to children. A bowl of ~100 to 150g cooked GR (50g dry weight) can provide ~60% of the Chinese Recommended Nutrient Intake of vitamin A for 6- to 8-yr-old children. Tang *et al.* (2012) have actually shown that the translation of ß-carotene in GR2 into vitamin A was better than expected. Nevertheless, the activists have exploited a deficiency in seeking consent from the parents of the kids who participated in the trials (Feng 2012a), and castigated the whole lot of the trials, though the deficiency has nothing to do with the science behind the trials. The response of the Chinese Government acting against the Chinese scientists and paying compensation to the parents of the children involved in the trials, has made matters worse (Feng 2012b, Liu 2015). The crisis was compounded with the journal retracting the paper by Tang *et al.* (2012), primarily on account of the parental consent issue (see American Journal of Clinical Nutrition, 2015, vol. 102, pp. 715).

In order to reduce the cost burden on the farmers and the consumers, the 'Golden Rice Humanitarian Board' facilitates cost free technology transfer to the developing countries. India, Bangladesh, Philippines and China which would benefit most from this arrangement have not been able to take advantage, in spite of the fact that these are the countries that need GR most to save millions of women and children from VAD disorders, particularly blindness and death.

Potrykus (2010), Beyer (2010), Dubock (2013, 2014) and Kameswara Rao (2005, 2013, 2015), have discussed the contingent issues, analysing the causes for the impasse. This is a clear case of science and technology subdued by politics and activism, which totally relied up on an extreme, but wholly illogical, interpretation of the Precautionary Principle (Kameswara Rao 2015). Wesseler and Zilberman (2014) have calculated that the delay over the last 10 years in making GR available has caused losses of at least 1,424,680 life years for India, ignoring indirect health costs of VAD which can be US$199 million per year, all of which demonstrate the economic power of the opposition towards GR. Ropeik (2014) asserted that GR opponents should be held accountable for health problems linked to VAD.

More and more information on GR has been flowing in on a daily basis, most of which is available at www.goldenrice.org.

3.7.2.3 Golden Sorghum

The Africa Biofortified Sorghum (ABS) Project, in collaboration with DuPont-Pioneer, developed the world's first sorghum transformation system as well as the world's first golden sorghum (Wambugu *et al.* 2012, Anonymous 2014). This transgenic sorghum has elevated levels of 5.7-21μg/g of β-carotene. The successful development of golden sorghum has put on hold all other vitamin A interventions in Kenya and Nigeria (Wambugu 2014).

3.7.2.4 Orange Maize

The orange maize (Pixley *et al.* 2013) was found to be as efficacious as vitamin A supplementation in a community-based, randomised placebo-controlled trials involving rural Zambian children even in the presence of high liver reserves of vitamin A (Gannon *et al.* 2014). In this trial involving 133 children, orange maize delivered 17-24μg/g of β-carotene when consumed as a staple food without causing hypervitaminosis A which was observed with the use of pre-formed vitamin A from supplementation and fortification (Gannon *et al.* 2014).

3.7.3 Folate (Vitamin B_9)

Folate is the collective name for tetrahydrofolate and its derivatives that constitute vitamin B_9, involved in the synthesis of the components of nucleotides, methionine, serine and glycine and in histidine catabolism (Blancquaert *et al.* 2010). The naturally occurring form is folate while the synthetic supplement is folic acid. Folate is needed for the maturation of red blood cells and for nerve function (Beers 2003), as well as for the synthesis of pantothenate (vitamin B_5). In plants folate is of primary importance in lignin, alkaloid, betaine, and chlorophyll biosynthesis, besides being essential for photorespiration (Patil and Kameswara Rao 2015).

Folate cannot be synthesised by humans *de novo* and the staple foods rice, wheat and potatoes are poor in folate. Hence we depend entirely up on dietary sources such as spinach, lentil, beans, oranges, yeast, eggs and liver that are rich in folate. Post harvest losses of folate are extensive as also losses during food processing (freezing 5%, cooking 70%, cook and drain 75%, reheat 30%). The RDA for folate is 400μg and the safe upper limit is one mg (Beers 2003).

Several diseases and disorders are linked to folate deficiencies. A low folate status and the occurrence of megaloblastic anaemia and neural tube defects, is well correlated. Many neurodegenerative disorders, including Alzheimer's disease, an increased risk of cardiovascular disease and development of a range of cancers have been linked to folate deficiency. Folate reduces blood levels of homocysteine, a byproduct of protein metabolism, associated with higher rates of cardiovascular disease and stroke (Nestle 2002). For these reasons, folate biofortification attracted special attention, and several scientists believe that folate biofortification through GE should be given priority (Bekaert *et al.* 2007, Newell-McGloughlin 2010, Beyer 2010, Dubock 2013).

GE tomatoes accumulated up to 840μg of folate per 100g (Diaz de la Garza *et al.* 2004, 2007), which is more than sufficient to provide the daily dietary requirement. A transgenic rice with two genes from *Arabidopsis thaliana* accumulated a 100 times more foliate (1,723μg/100g) than in the wild type, with 100g of polished raw grains containing up to four times the adult daily folate requirement (Storozhenko *et al.* 2007). Naqvi *et al.* (2009) doubled folate content (200μg/100g) in a transgenic corn, which also contains stacked genes for ascorbic acid and three vitamin A precursor carotenoids.

Contingent issues related to folate biofortification were discussed in detail (Bekaert *et al.* 2007, Blancquaert 2010). There is extensive information on the biosynthesis of folate in plants to envisage promising novel folate fortified crops (Scott *et al.* 2000, Bekaert *et al.* 2007), more particularly wheat, banana and potato. It was observed that folic acid supplementation alone may not succeed in reducing the incidence of neural tube defects, where as folate fortified grain products did result in a significant reduction in their incidence (Beyer 2010), which highlights the importance of the biofortification route. Excessive folate consumption may interfere with detection of B_{12} deficiency (Nestle 2002) and hence both should be taken together. Blancquaert *et al.* (2014) recommended folate biofortification of highly consumed crops over folate supplementation as high folic acid intake could have adverse effects on human health such as an increased risk of prostate and colorectal cancer (Cole *et al.* 2007). Over all, folate biofortification of staple crops should be a valuable, complementary and cost-effective intervention in fighting folate deficiency worldwide, more so in the poor countries.

3.7.4 Ascorbic Acid (Vitamin C)

L-Ascorbic acid (vitamin C) is an essential nutrient, not synthesised in the human body, but must be obtained from a diverse range of plant foods such as citrus fruits, gooseberry, tomatoes, cabbage, drum stick, leafy vegetables and others.

Vitamin C is needed for, (a) the formation of blood vessels, (b) bone and connective tissue, (c) wound healing, (d) promoting absorption of iron, and (e) as an antioxidant (see Section 3.9). A deficiency of vitamin C leads to scurvy, characterised by anaemia, spongy gums, and subcutaneous bleeding, which can be lethal. Vitamin C is now believed to prevent chronic diseases such as (a) heart disease, (b) stroke, (c) some types of cancer, and (d) several neurodegenerative diseases (Hancock and Viola 2005). However, evidence for vitamin C's protective effect against cataracts is the strongest (Beers 2003). The RDA of vitamin C is 90mg and 35mg more for smokers. Being water-soluble, vitamin C is almost non-toxic, with the safe limit at 2,000mg. As vitamin C does not accumulate and cannot be stored in the body, it needs to be continuously supplied and is among the most widely supplemented nutrients, in different forms and added to diverse prepared foods.

Probably in view of wider availability of vitamin C in different foods and as industrial supplements, only a few attempts were made for its biofortification. The levels of vitamin C were enhanced two to four times in transgenic maize kernels (Chen *et al.* 2003) and in strawberry (Agius *et al.* 2003). Naqvi *et al.* (2009) used the rice dehydroascorbate reductase (DHAR) cDNA to transform maize and obtained a six-fold increase in ascorbate. Interestingly, this elite inbred M37W maize from South Africa is gene stacked also for folate and pro-vitamin A carotenoids, along with vitamin C.

There has been substantial progress in the understanding of vitamin C biochemistry in plants with a number of structural genes cloned (Hancock and Viola 2005, Laing *et al.* 2007, Stevens *et al.* 2007; Naqvi et al. 2009, Cruz-Rus *et al.* 2011, Kondo *et al.* 2015) and this should encourage efforts to enhance vitamin C in both existing and new plant food sources.

3.7.5 Tocopherols (Vitamin E)

Tocopherols and tocotrienols constitute vitamin E. In plants tocopherol occurs in different isoforms, α-tocopherol and γ-tocopherol being the most common. While α-tocopherol and its derivatives are considered to be the most beneficial dietary forms, γ-tocopherol predominates in many foods. Vitamin E is commonly available from many plant foods, more particularly vegetable oils, wheat germ, leafy vegetables, legumes, and others. Consequently vitamin E deficiency condition, often arising out of a low fat intake, is not very common.

Vitamin E is a fat-soluble compound and so is absorbed better in the presence of fats, but accumulates in the body, excess amounts being harmful as they may lead to haemorrhage and stroke (Beers 2003). The RDA of vitamin E is around 15mg, with the safe limit at 1,000mg.

A wide range of health benefits are attributed to vitamin E. Numerous vitamin E supplements and skin creams are widely used in the belief that vitamin E promotes skin health. However, many studies failed to provide any conclusive proof for vitamin E preventing cancer or coronary heart disease or other disorders it is supposed to prevent (Haber 2006). Currently, most references cite vitamin E as only an important dietary antioxidant (Beers 2003) (see Section 3.9).

There have been a few GE products with enhanced levels of tocopherols. Shintani and Dellapenna (1998) enhanced α-tocopherol levels in *Arabidopsis* using α-tocopherol methyl transferase gene. Rocheford *et al.* (2002) used a similar approach to modify the ratio of α-tocopherol to γ-tocopherol in corn and identified molecular markers to assist breeders. Additional insights into tocopherol biosynthesis (Porfirova *et al.* 2002, Takami *et al.* 2002, Cahoon *et al.* 2003) should be useful in enhancing levels of tocopherols in other food crops. If this is really necessary at all, is a different question.

3.7.6 Multi-vitamin Corn

Naqvi *et al.* (2009) achieved an intellectual feat of stacking five genes in an elite maize inbred M37W from South Africa, to simultaneously provide for three vitamin A precursor carotenoids (α- and β-carotenes and β-cryptoxanthine) as well as folate and ascorbate in the endosperm. The kernels of this transgenic maize showed 169-fold increase in β-carotene levels, double the normal amount of folate and a 6-fold increase in ascorbate (Naqvi *et al.* 2009). The stability of the enhanced levels of these vitamins was checked up to the T3 generation. This impressive achievement opened up opportunities for the simultaneous enhancement of levels of several nutrients in a single crop (Patil and Kameswara Rao 2015). Golden sorghum detailed above, is another example of multi-nutrient enrichment of a single crop, to deliver higher levels of β-carotene, lysine, iron and zinc (Wambugu *et al.* 2012; Anonymous 2014).

3.8 Enhancement of Levels of Mineral Elements

3.8.1 Role of Mineral Elements in Human Health

The area of study of the role of inorganic chemical elements in the metabolic processes of diverse organisms has now come to be recognised as '*bioinorganic chemistry*'. In the biological systems, sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), chloride (Cl) and phosphate (P) are the macrominerals, as they are needed in large quantities. The macrominerals also function as electrolytes in the human system, to regulate nerve and muscle function, acid-base balance and the volume of fluid-contained in cellular compartments and tissues (Beers 2003). Copper (Cu), fluoride (Fl), iodine (I), iron (Fe), selenium (Se) and zinc (Zn) are involved in very important metabolic reactions but are needed only in very small quantities (microminerals). Basing on their critical metabolic roles, paucity in common plant foods and the disorders their deficiency causes in human populations, iron, zinc, calcium, selenium, iodine, and fluoride, are regarded as essential minerals.

Organisms obtain mineral elements from the environment and cannot synthesise them. Plants absorb them from soil and water while animals get them from water and plant and animal foods. Mineral content in plants largely depends upon the availability in the soil. If the soils are deficient in any mineral, plants also suffer from mineral deficiencies, exhibiting symptoms that are often confused with disease symptoms. Mineral enrichment of the soils is often necessary as many soils are deficient in one or the other mineral element (Dai *et al.* 2004). Soil application of inorganic nutrients is a common practise in agriculture. The application of nitrogen, phosphorus, potassium and sulphur fertilisers generally increases crop yield and nutritional quality but excessive fertiliser application can result in undesirable increases in nitrite, nitrate, titratable acidity and acid to sugar ratio, while decreasing the concentration of vitamin C, soluble sugar, soluble solids, magnesium and calcium in some crops (Wang *et al.* 2008). The often suggested foliar application is rather impractical when thousands of hectares of crop fields are involved.

Mineral elements may accumulate in plant parts other than those that are usually consumed. The mineral content of plant and animal foods particularly in the storage organs used as food is often low as it is only the metabolic surplus. For these reasons, the quantity of different minerals obtained from plant and animal foods is uncertain. Cognitive development and disease resistance in children are severely affected even by mild levels of mineral malnutrition which would enhance childbirth mortality. For a long time, people have been advised to enhance their mineral intake through supplementation, in the form of tablets, capsules, water and drinks. This approach has worked largely well in ameliorating deficiency of iodine (iodised salt) and fluoride (toothpaste, water), but either government policy (iodised salt) and/or market forces (fluoridated products) compel even those people who do not need these elements to use them. During the past couple of decades there has been an increasing awareness of the need to enhance the mineral content particularly calcium, iron and zinc in plant foods, because of the inadequacy of industrial fortification and supplementation.

The RDAs of most minerals needed for maintaining health in adults have been determined. Either a deficiency or an excess intake of any of the minerals will result in nutritional disorders. People who eat a balanced diet are unlikely to develop major mineral deficiencies, except probably for iron or iodine (Beers 2003). Mineral deficiency disorders are most prevalent among the poor and particularly those in developing countries, for want of adequate and appropriate foods. They may also arise when diets are predominantly based on staple foods, such as milled cereals, which are low in bioavailable mineral content (Christou and Twyman 2004), or among vegetarians and vegans who eat restricted diets (Beers 2003).

Notwithstanding the crucial roles the minerals play in our health, most minerals are toxic on continued intake at levels far and above RDAs (Beers 2003). If mineral elements are not readily metabolised they accumulate (bioaccumulation) building up to toxic concentrations. Crop plants also absorb heavy metals from soils which may bioaccumulate to potentially toxic levels. Fu *et al.* (2008) reported high levels of 10 heavy metals (arsenic, barium, cadmium, cobalt, chromium, copper, mercury, manganese, nickel and lead), accumulating in rice grown near E-waste recycling areas in China and the consequent risk to human health. An unsupervised consumption of megadoses of even the essential minerals such as copper and manganese, may lead to toxicity (Beers 2003). The kidneys take the major load from an excess intake of minerals as they filter the electrolytes in the blood and excrete the excess mineral quantities. A disturbance in the balance of electrolytes leads to many health disorders.

Goudia and Hash (2015) suggested that the combination of conventional breeding with modern GE approaches and quantitative trait loci (QTL) analysis is important for developing crop cultivars with enhanced micronutrient concentrations to improve human health. As plants can obtain mineral elements only by absorbing them from the soil, the sole means of enhancing mineral content in plant foods is by redirecting one or more reactions to improve the efficiency of uptake and transport of the respective minerals into the edible tissues leading to their accumulation, as was done for calcium in carrots (Morris *et al.* 2008, Connolly 2008). A strategy to increase the bioavailability of the minerals present in the plant foods is by reducing phytate and/or enhancing phytase (Frossard *et al.* 2000, Colangelo and Guerinot 2006), as was done in soybean

(Denbow *et al.* 1998), alfalfa (Austin-Phillips *et al.* 1999), wheat (Brinch-Pedersen *et al.* 2000) and maize (Drakakaki *et al.* 2005) (see Section 3.10.2). The products and strategies developed to deliver higher quantities of the essential minerals are detailed hereunder.

3.8.2 Iron (Fe)

The crucial importance of iron as a nutrient was recognised a very long time ago in the context of haemoglobin in the red blood cells that carries oxygen and delivers it to the tissues, but not so much in the context of it being essentially required for the formation of many proteins and enzymes. Iron is a redox-active constituent of the catalytic site of heme and non-heme iron proteins (Beyer 2010), such as haemoglobin, myoglobin, leghaemoglobin, cytochromes, nitrogenase and many other enzymes.

We obtain almost all of iron needed from food, which contains two types iron: heme iron from animal foods (red meats, poultry, fish, kidneys and liver) and non-heme iron from plant foods (soybean products, legume grains, leafy and other vegetables). Encapsulated iron (as fumarate or sulphate) and iron fortified foods, drinks and mineral water provide non-heme iron. Heme-iron is absorbed much better than non-heme iron, but non-heme iron accounts for more than 85% of iron in the average diet. Less than 20% of the consumed non-heme iron is absorbed into the body, but is absorbed better when it is consumed along with animal protein and vitamin C (Beers 2003).

By and large the staple foods (cereals, millets and tuber crops) consumed by the poor, particularly in the developing countries, are low in iron content. These populations cannot either access or afford the other foods that may provide adequate amounts of iron. Consequently, they suffer from iron deficiency, which is one of the most prevalent and serious nutritional disorders. More than one-third of the world's population suffers from iron deficiency leading to low haemoglobin and anaemia (WHO 2001). Deficiency of iron adversely affects cognitive development, resistance to infection, work capacity, productivity, and pregnancy. Children of anaemic mothers have low iron reserves, requiring more iron than is supplied by breast milk, and suffer from impaired physical growth and irreversible defective mental development (Newell-McGloughlin 2010, Dubock 2013). Annually, about 800,000 deaths are attributable to iron deficiency. Iron excess happens when iron accumulates in the body from excessive iron therapy which may affect the coronary arteries (Beers 2003).

There is no regulated physiological means of excreting iron from the human body. Only small amounts of iron are lost daily due to mucosal and skin epithelial cell sloughing, which is replaced by 1 to 2 mg of iron absorbed from food each day.

The levels of ferritin, an intracellular metallo-protein involved in the storage and controlled release of iron, are taken as a clinical indication of total amount of iron in our body. One unit of ferritin holds 1140 iron ions. Low ferritin levels are a reflection of low levels of iron. Both low and high ferritin levels have their own health implications. Based upon extensive comparative data, RDAs for iron intake have been calculated (US National Research Council 2000). Nevertheless, there are different estimates of 'normal' ferritin reference levels in the serum, which vary among human age groups, from 18 to 270 *n*g/ml in men and 15 to 160 *n*g/ml in women. Beers (2003) cited RDAs for iron at 8mg for normal adults, 18mg for pre-menopausal women, 27mg for pregnant women and 9mg for breastfeeding women, with an upper safe limit at 45mg. How much of this intake is absorbed and used by the body system depends upon a number of factors some related to the general health of the individual.

The pressing need to enhance levels of iron intake was recognised over half-a-century ago, and large-scale supplementation programmes were implemented in different parts of the world. But these efforts have not reached the poor. Enthused by the success of iodine fortified salt in reaching the poor in remote parts of almost every country, the National Institute of Nutrition, Hyderabad, India, proposed a double fortified salt, salt with both iodine and iron, to mitigate iron deficiency in large sections of populations in the developing countries (Sivakumar and Nair 2002). This product is now being marketed in India.

There have been a few efforts to develop iron rich varieties of plant foods. The ICRISAT has released a high-iron pearl millet variety ICTP 8203Fe, named *Dhanshakti*, developed through conventional selection and breeding, with 9% higher iron content than in the parent variety ICTP 8203 (ICRISAT 2013).

The uptake and accumulation of iron in plants is complex and developing iron-rich GE varieties is fraught with difficulties. Nicotianamine is a metal complex which chelates Fe(II) and mobilises it further to other parts of the plant (Grotz and Guerinot 2006) and plants engineered to express nicotianamine showed double the accumulation of both iron and zinc. The 'cross-talk' between the pathways of accumulation of iron and zinc affects enhancement strategies of both. As transferrin also transports iron along with other minerals, excessive calcium and iron reduce zinc absorption, and vice versa (Beers 2003). Nonetheless, several promising iron-rich crops have been developed, though almost none of these is closer to commercialisation. The following are some representative examples of iron-rich crops:

a) **Rice:** Ishimaru *et al.* (2006) have shown that in addition to absorbing an Fe3+-phytosiderophore, rice possesses a novel Fe-uptake system that directly absorbs the Fe2+, a strategy that is advantageous for growth in submerged conditions. This makes it a little easier to enhance iron content in rice than in other crops.

   The iron content of the rice endosperm varies from 1 to 8μg/g, in different cultivars. Although 6μg/g of iron in rice has a positive impact on nutritional status (Haas *et al.* 2005), significantly higher levels are considered desirable (Beyer 2010). Due to the lack of adequate variability in iron content of rice seed, only the transgenic approach is capable of increasing iron partitioning in favour of the grains. About 39 genes are thought to control iron homeostasis in rice (Gross *et al.* 2003), of which the rate-limiting ones are currently unknown (Beyer 2010).

   Expression of ferritin causes a three- to four-fold increase in iron levels in rice endosperm (Goto *et al.* 1999, Goto *et al.* 2000, Lucca *et al.* 2002, Vasconcelos *et al.* 2003). Iron accumulation was enhanced in rice by overexpression of the barley *naat-A* and *naat-B* genes encoding nicotianamine aminotransferases (Takahashi *et al.* 2001). Cheng *et al.* (2007) enhanced iron in rice using a mutant gene for nicotianamine aminotranferase.

   By continuously transcribing a gene at a higher level than normal, Wirth *et al.* (2009) and Johnson *et al.* (2011), have raised the levels of both iron and zinc in the endosperm of transgenic rice Although polishing of rice grains leads to a reduction in mineral levels, ferritin-enhanced transgenic rice still retains high levels of iron in the polished rice grains.

b) **Maize:** The removal of anti-nutrients from plant foods can enhance the bioavailability of mineral content. A combined strategy of using an endosperm-specific co-expression of recombinant soybean ferritin and Aspergillus phytase has resulted in significant increases in the levels of bioavailable iron in maize (Drakakaki *et al.* 2005).
c) **Wheat:** The positional cloning of a quantitative trait locus (QTL) "Gpc-B1", associated with enhanced grain protein content and coding for a NAC transcription factor (in three different domains) that regulate senescence, simultaneously improved grain protein, and zinc and iron content in wheat (Uauy *et al.* 2006) and plants engineered to express nicotianamine showed double the accumulation of both iron and zinc.
d) **Barley:** The pathways of iron and zinc transport seem to be interlinked as the plants engineered for iron also showed increased levels of zinc (Grotz and Guerinot 2006). Increase in iron, along with zinc, was demonstrated through continuously transcribing a single gene at a higher level than normal in barley (Ramesh *et al.* 2004).
e) **Sorghum:** The golden sorghum from the Africa Biofortified Sorghum Iniative, is a stacked transgenic for enhanced levels of β-carotene, iron and zinc, which is now under field trials (Wambugu *et al.* 2012, Wambugu 2014, Anonymous 2014).
f) **Lettuce**: Ferritin genes in a transgenic lettuce enhanced iron accumulation and growth as well (Goto *et al.* 2000).
g) **Banana:**The technology for a transgenic banana with high iron content developed in Australia is being transferred to India (Dubock 2013).

There is a long way to go before iron-rich plant foods get on to the table of all those who need them.

3.8.3 Zinc (Zn)

Zinc (Zn), the most abundant element after iron, is essentially needed by diverse groups of organisms, for the structural integrity and activity of over 100 enzymes of all classes, including those involved in the synthesis of DNA and RNA. Zinc is also found in the side chains of several amino acids. Zinc readily binds to many proteins and is an important component of 'zinc finger proteins' (see Section 3.3.5), the small protein motifs which stabilise the structure of a large numbers of other proteins and constitute parts of transcription factors.

In the human body, most of zinc is present in the brain, muscle, bones, kidney, liver, eye, prostate and semen, and is involved in (a) signalling in cell communication, (b) insulin formation, (c) healthy healing of skin and muscle wounds and growth and (d) enhancing immunity (Beers 2003).

Beers (2003) suggested 15mg as the RDA of zinc. The U.S. NRC's RDAs of zinc are 8mg/day for women and 11mg/day for men (US NRC, 2000). Zinc in cereals and grain-legumes is inexpensive and as easily absorbed as from the expensive commercial fortification, which is by zinc sulphate or zinc gluconate. As zinc interacts with other micronutrients it is not administered alone, but along with other encapsulated nutrients.

Excess intake of zinc is uncommon, but may result from excessive consumption of foods and drinks packaged in zinc lined containers. Zinc is toxic on continued intake at over and above the RDA, but the safe upper limit is not very clear at present. Excess zinc curtails the absorption of copper.

Insufficient dietary intake or low availability of zinc and/or high phytic acid in the food consumed, cause zinc deficiency disorders. More people (3.2%) in the world suffer from zinc deficiency than from any other mineral deficiency. The majority of people in the developing countries, particularly the aged and the children, suffer from zinc deficiency, which causes a wide variety of ailments from cold to diarrhoea, affects the nervous system, lowers immunity and causes over half-million child deaths annually (Patil and Kameswara Rao 2015).

Given the adequate presence of zinc in crop fields, we should be able to obtain adequate quantities of zinc from many plant foods such as cereals, beans, sesame, mustard and so on, but this does not happen all the time. The factors that restrict the availability of zinc to the body system are (a) much of the zinc consumed in the diet is not absorbed, (b) vegetarian diets contain large amounts of phytic acid that affects the availability of absorbable zinc (see Section 3.10.2) and (c) large amounts of calcium and iron may reduce zinc absorption (Beers 2003), as transferrin also transports iron, and excessive iron reduces zinc absorption, and vice versa. Though red meats, oysters, lobster, and other non-vegetarian foods are projected as containing high concentrations of zinc, non-vegetarians also suffer from zinc deficiency as much as vegetarians, for similar reasons. Zinc absorption would be enhanced if phytic acid levels in the food are reduced (Adams *et al.* 2002).

There have been attempts to select crop varieties with higher zinc content in conventional breeding, but the success mainly depended upon the levels of zinc in the soils. Zinc deficient soils are common in many countries such as Turkey, India, China, Australia, etc., which prompted soil application or fertigation of zinc, to raise soil zinc levels. Cakmak (2008) compared different approaches to enhance zinc levels in the edible parts of crop plants and favoured foliar or combined soil and foliar application of zinc fertilisers as a highly effective and very practical way to maximise uptake and accumulation of zinc in whole wheat grain, raising zinc's concentration up to 60 mg/kg.

Palmgren *et al.* (2008) discussed the problems and solutions related to biofortification of zinc in cereals, and suggested that zinc can be fortified in cereals and grain-legumes inexpensively as an absorbent. However, as the root-shoot distribution of zinc is controlled mainly by toxic heavy metal transporters, it is cautioned that heavy metals may also accumulate along with zinc. Heavy metal uptake from the soil by food crops is a distinct possibility (Fu *et al.* 2008). The following situations suggest promising possibilities of enhancing zinc (and iron) concentrations in food crops:

a) There is substantial genetic variation in zinc composition not only between different crops but also among different varieties of a crop (Broadley *et al.* 2008), which can be exploited for biofortification;
b) A significantly higher variability was found in zinc content of polished rice grains making this trait a likely candidate for precision breeding (Beyer 2010);
c) The pathways of iron and zinc transport seem to be interlinked as the plants engineered for iron also showed increased levels of zinc (Grotz and Guerinot 2006). Increase in iron and zinc were demonstrated through continuously transcribing a single gene at a higher level than normal in barley (Ramesh *et al.* 2004) and rice (Johnson *et al.* 2011);
d) Gelin *et al.* (2007) identified a gene locus associated with seed zinc accumulation in navy bean. Overexpression of a zinc transporter enhanced zinc content in barley seed (Ramesh *et al.* 2004);

e) The positional cloning of a QTL "Gpc-B1" associated with enhanced grain protein content and coding for a NAC transcription factor (in three different domains) that regulate senescence, improved grain protein, and zinc and iron content in wheat (Uauy *et al.* 2006) and plants engineered to express nicotianamine showed double the accumulation of both iron and zinc; and
f) The golden sorghum from the Africa Biofortified Sorghum Iniative, is a stacked transgenic for enhanced levels of β-carotene, iron and zinc, which is under field trials (Wambugu *et al.* 2012, Wambugu 2014, Anonymous 2014)

On the other side, zinc enriched grains contribute to crop productivity as they result in better seedling vigour, denser stands and higher stress tolerance on potentially zinc deficient soils (Cakmak 2008).

3.8.4 Calcium (Ca)

Calcium (Ca) is needed throughout one's life as it is critical in building stronger and denser bones and teeth. Calcium regulates dopamine and prevents age related brain disorders. It is also needed for normal muscle function and to maintain heart rhythm (Beers, 2003). About 95% of the body's calcium lies in the bones and teeth and the rest is involved in metabolic functions. Long-term calcium deficiency may lead to rickets, poor blood clotting, osteoporosis in post-menopausal women and a higher risk of bone fractures. Both absorption and functions of calcium require vitamin D.

The RDA of calcium varies through one's life from 200 mg/day in infants to 1,000-1,300mg/day in the older (Beers 2003). Calcium is obtained from mother's milk by infants and from dietary sources by others. Dairy products are rich in calcium containing about 1.5 mg/g. As some individuals cannot consume dairy products due to health reasons (lactose intolerance) or religious beliefs (vegans), a large part of global population depends upon plant foods for calcium. Though calcium is far safer than many other minerals, excessive intake of over 2,500 mg/day is ill-advised (Beers 2003).

The importance of oral supplementation of calcium appears to have been recognised centuries ago, as reflected by traditional practises of using calcium hydroxide in food processing and in masticatories. '*Paan*' is composed of betel-nut (*Areca catechu*) pieces and powdered *cutch* (bark of *Acacia catechu*) along with several flavourings and sweeteners, all wrapped in betel-pepper (*Piper betle*) leaves smeared with slaked lime (calcium hydroxide). Chewing *paan* and/or tobacco leaves smeared with calcium hydroxide, is prevalent in the Asian and Oceanic countries, particularly India, Pakistan and Bangladesh, cutting across socio-economic and religious barriers, on par with coffee and tea. Native Americans and Mexicans also chew calcium hydroxide smeared tobacco. Calcium hydroxide is used in some Thai foods and is used in Mexico to treat corn for tortillas.

The problem of calcium deficiency is related more to bioavailability and bioconversion, rather than virtual intake in food. A number of plant foods, such as sea weeds, tree nuts, ladies' fingers, kale, leaves of drumstick tree, beans, broccoli, oatmeal, molasses, soya milk, tofu and so on, are identified as rich sources of calcium, as also salmon and sardines. Soybean is among the richest sources, with 3.5 mg/g of calcium. In some leafy plant foods such as spinach and amaranths, calcium primarily exists as a complex bound to oxalate, phytate, fatty acids, proteins and other anions (Franceschi and Nakata 2005) and so not easily available to the body system. Calcium absorption appears to be inversely proportional to the oxalic acid content in the food and phytic acid also reduces the bioavailability of calcium (Raboy 2002, Hambidge *et al.* 2005).

Food-grade calcium carbonate or calcium phosphate is commonly used in commercial fortification, dispensed as tablets or in combination with other encapsulated nutrients. Calcium carbonate is also added to drinks such as soy milk (though it has more calcium than milk) and orange juice. The recommended supplementation dosage is 600mg/day, as a higher intake of calcium reduces its absorption.

Rovensky *et al.* (2003) have demonstrated that powdered eggshell taken in food or water, was effective in the prevention and treatment of osteoporosis in post-menopausal women. The bioavailability of egg shell calcium compared well with that of food grade calcium carbonate and had positive effects on bone and cartilage (Patil and Kameswara Rao 2015).

In the face of failure of a few conventional breeding efforts to produce any appreciable calcium rich foods, Connolly (2008) and Morris *et al.* (2008) have developed a transgenic carrot with high calcium content (27 to 29mg/100g). In feeding trials on rats and humans, these carrots delivered 41% more calcium than the controls. Transgenesis for high calcium did not show any adverse effect on the growth, development, or fertility of the crop. This was achieved by using a modified calcium/proton anti-porter transgene (sCAX1) to increase calcium transport into vacuoles. Even in these carrots, a fraction of calcium was bound to anti-nutrients. Nevertheless, similar protocols are likely to result in high accumulation of other mineral elements in crops.

3.8.5 Selenium (Se)

Selenium (Se) is ubiquitous in soils, but exists mainly in insoluble forms in high-Fe, low-pH and certain leached soils, and hence is often of limited availability to plants (Lyons *et al.* 2003). Selenium is essentially needed for cellular functions, but only in trace amounts. In living systems, selenium is found in the amino acids selenomethionine, selenocysteine and methylselenocysteine. Selenium works along with vitamin E (Beers 2003) and is a component of antioxidant enzymes glutathione peroxidase and thioredoxin reductase both in plants and animals (Hartikainen 2005). Selenium is a co-factor in de-iodinase enzymes which convert one thyroid hormone into another and so is an essential micronutrient in every cell that uses thyroid hormone.

We usually get adequate quantities of selenium from foods such as nuts, cereals, meat, mushrooms, fish and eggs. There may be regional differences, as for example, the Australian wheat appears to contain selenium at far above the global average levels (Lyons *et al.* 2003).

Implicated in cancer, diabetes, HIV/AIDS, tuberculosis, selenium is suggested as a chemo-preventive of these diseases. Finley *et al.* (2001) have shown that selenium in high-selenium broccoli protected Sprague-Dawley rats from mammary and colon cancers, as selenium from high-selenium garlic reduced chemically induced mammary tumours.

Disorders caused by selenium deficiency are a possibility but not of wide occurrence, even in New Zealand and Finland with low selenium intake (Beers 2003). Selenium deficiency results in an under-active thyroid or causes other thyroid related complaints. Selenium deficiency is also implicated in osteoarthritis, rheumatoid arthritis, muscular degeneration, hay fever, infertility, cataract, etc. In China selenium deficiency occurs in association with Keshan, a viral disease, which may damage the heart (Beers 2003). Selenium deficiency may result in heart disease due to impaired antioxidant activity.

The RDA of selenium is about 55μg/day with 400μg as the upper limit (Beers 2003, Johnson 2012). Selenium is toxic in high concentrations causing selenosis and other disorders (Johnson 2012) and hence an excessive and/or continued intake of selenium is ill-advised.

Selenium is available as a commercial supplement but is not yet a routine component of encapsulated nutrient supplementation, nor were there any attempts to enhance selenium levels through breeding. Despite several publications on the importance of selenium in animal and human health, as at present selenium augmentation in foods and/or its supplementation have not received any serious attention, except for some suggestions that the high-selenium Australian wheats should be used to build up stocks for conventional breeding (Lyons *et al.* 2003).

3.8.6 Iodine (I)

Iodine (I) is essential for life but is needed only in traces. A large proportion of iodine, in the form of salts or complexed with other compounds, lies in the tissues such as the mammary glands, eyes, mucosa, arterial walls, the salivary glands and so on. Most of the iodine in the body occurs in the thyroid glands (Beers 2003) as 60% of the thyroid hormones is iodine.

Iodine is absorbed from the blood by the thyroid gland which synthesises two thyroid hormones, thyroxine (T4) and tri-iodothyronine (T3), and releases them into the blood. This process is regulated by the thyroid stimulating hormone (TSH) synthesised in the pituitary gland. Iodine is a constituent of T3 and T4, which are synthesised from the amino acid tyrosine. Selenium is needed to convert T4 to the active hormone T3 and vice versa. Thyroglobin is an iodine containing protein that stores T3 and T4. The basal metabolic rate is regulated by thyroid hormones which function at gene transcription stage.

In India, 42 million people suffer from thyroid disease and about 100 million suffer from subclinical (not apparent in routine diagnosis) disease (Biospectrum, June 24, 2013). Iodine insufficiency, often resulting in the enlargement of thyroid gland, adversely impacts physical and mental development, those affected being known as 'cretins' (Dubock 2013). Iodine deficiency results in hypothyroidism which reduces the basal metabolic rate even by 50% and a restored thyroid function would greatly enhance the basal metabolic rate. Hypothyroidism causes a set of symptoms mainly characterised by fatigue, goitre, low body temperature and weight gain. Children, and pregnant and lactating mothers are at a higher risk from low iodine intake than others.

As per the data of the U.S. National Research Council (2000), 110 to 130 μg/day of iodine is required by children, 150 μg/day by adults, while pregnant women and lactating mothers require 220 and 290 μg/day, respectively. As a number of different organs require iodine for their functioning, higher doses of supplementation are often recommended. However, excessive intake of iodine may result in hyperthyroidism, in certain individuals.

Iodine is sourced by us from sea foods, water and plant foods from coastal areas and sea salt. In countries where seafoods form a significant component of the diet as in Japan, a far higher quantity of iodine is consumed than in other places.

Iodine deficiency is a serious problem in land locked and/or hilly regions and countries. Iodine supplementation has cured thyroid deficiency syndromes. In Europe, iodine supplementation through sea salt to cure goitre, that involves an enlargement of the thyroid gland, dates back to the Middle Ages. Zimmermann *et al.* (2006) reported a significant improvement of cognition on iodine supplementation in iodine-deficient children in Albania.

Elemental iodine in blood is toxic. Iodine containing products such as iodised salt or tincture of iodine or iodine rich seafoods, may cause hypersensitivity in some individuals, but mistaken for allergy, which is an immunological reaction to certain proteins, in some individuals.

Commercial supplementation of iodine is through iodised salt and potassium iodide encapsulated along with other nutrients. With governmental support for iodised salt in two-thirds of the world, non-iodised salt is hard to get, except in rural and un-regulated markets. Even so, a large segment of the global population is still iodine deficient and suffers from hypothyroidism syndrome which can be remedied by promoting awareness about the importance of iodine supplementation.

As the current interventions being generally adequate, not much attention was paid to enhancing iodine content of food plants. Dai *et al.* (2004) developed iodine-enriched vegetables by iodate application to soil.

3.8.7 Fluoride (Fl)

Fluoride (Fl) has been recognised for long as the most important means of preventing dental decay and strengthening the bones. While most people get adequate amounts of fluoride through water, tea, coffee, milk, and most plant foods (Kameswara Rao 2000), small sections of populations in different parts of the world suffer from fluoride deficiency. There is a massive commercial supplementation programme dispensing fluoride in toothpastes, gels, table salt, water, etc. In the United States, prevention of dental caries through fluoridation is recognised as one of the 10 great public health achievements of the twentieth century. Though the optimal dosage is 1 to 2 μg/L of water, the recommended RDA of 3 to 4mg/day, with an upper limit of 10mg/day (Beers 2003) may work out to be a little in excess. It looks that a very large number of people consume more of fluoride than is needed. A continued intake of massive doses of fluoride would cause fluorosis, the weakening the teeth and the bones (Beers 2003). Nevertheless, fluoridation in the form of sodium fluoride or sodium hexafluorosilicate, has come to stay in most countries. There has been a severe criticism on the administration of large doses of fluoride on a massive scale and that too to people who do not need it. The commercial angle in this process, which provided impetus to administer via commercial supplementation on a massive scale, other important nutrients or chemicals that protect us from disease, is also a sour point (Nestle 2002). Against this background, there is no need to breed crops with higher fluoride content.

3.9 Enhancement of Antioxidants

3.9.1 The Antioxidants

Normal cellular metabolic activity routinely generates a large number of atoms with unpaired electrons called ‘free radicals’, as by-products. These highly reactive radicals interact with important cellular components such as DNA and/or cell membranes, seriously impairing cellular functions, even leading to cell death.

Free radicals are believed to contribute to many disorders such atherosclerosis, cancer, lung disorders, common cold, eye cataracts and memory loss (Beers 2003). Antioxidants protect cells against damage from free radicals. Antioxidants are molecules which interact with free radicals preventing damage to vital molecules. There are several enzyme systems within the body that scavenge free radicals. However, a number of micronutrients such as carotenoids (particularly β-carotene), anthocyanins, polyphenols, vitamins C and E, minerals like selenium and others are identified as antioxidants (Parr and Bolwell 2000, Lechman and Hamonz 2005). A regular consumption of polyphenols in plant foods has been associated with reduced risk of a number of chronic diseases, including cancer, cardiovascular disease and neurodegenerative disorders (Vauzour *et al.* 2010).

As the body cannot manufacture these micronutrient antioxidants they must come in the diet, particularly fruits and vegetables. It was shown that who eat adequate amounts of fruits and vegetables rich in antioxidants are less likely to develop heart disease and certain cancers (Nestle 2002). However, whether these benefits are due to the antioxidants or other substances in fruits and vegetables is not clear (Beers 2003). Nevertheless, a very large number of processed and prepared foods on the market have substantial amounts of added antioxidants, which also serve as preservatives. While RDAs are suggested for some compounds functioning as antioxidants in the context of their other functions, no RDAs are recommended for antioxidants *per se.*

A number of compounds with antioxidant activity have been identified in potato (Lachman and Hamouz 2005). The main potato antioxidants are polyphenols (*L*-tyrosine, scopolin, and caffeic, chlorogenic, cryptochlorogenic and ferulic acids), ascorbic acid, carotenoids, tocopherols, α-lipoic acid, and selenium. The red and purple potatoes contain acylated anthocyanins also. The pigmented potatoes display two to three times higher antioxidant potential in comparison with white-flesh potato. In tomato also several compounds such as lycopene, lutein, α-carotene, phenols, flavonoids, ascorbic acid and dehydro-ascorbic acid, are identified as antioxidants, exhibiting hydrophilic or lipophilic antioxidant activity (Ilahy *et al.* 2011).

Several studies have shown that daily consumption of fruits and vegetables increases plasma levels of the antioxidants which control blood pressure and reduce the risk of cardiovascular diseases and cancer. Interestingly, some of the antioxidants are likely to be useful as therapeutic agents. For example, there is substantial evidence that the isothiocyanates derived from sulphur containing glucosinolates are the principal source anticarcinogenic activity of Brassica vegetables (Richard *et al.* 2000) and the consumption of high glucoraphanin broccoli significantly reduced plasma LDL-C (Armah *et al.* 2015).

Recognising the diverse health benefits mediated via antioxidant activity of many secondary metabolites present in plant foods, a case was made for modifying and optimising the phenolic and other chemical content of food crops, both qualitatively and quantitatively, using diverse conventional and modern methods of plant breeding (Parr and Bolwell 2000). The more promising examples of enhancing chosen antioxidants in common plant foods are given hereunder.

3.9.2 Lycopene

Lycopene is a major antioxidant mainly found in tomato and is known for its potential to prevent prostate cancer. Several approaches have been used for enhancing lycopene content in fruits and vegetables. Natural high-pigment mutants of tomato are used in breeding strategies to alter lycopene levels in carrots (Santos and Simon 2002), which were also enhanced through genetic modification. Genetic manipulation enhanced lycopene levels in tomato (Rosati *et al.* 2000) and the expression of bacterial and yeast genes in transgenic tomatoes significantly enhanced lycopene levels (Mehta *et al.* 2002).

Lycopene being a fat-soluble pigment is poorly absorbed by the body from the fruits and vegetables. The *cis* or *trans* configuration of lycopene, may also affect absorption rates, as the *cis* form is more soluble and is preferentially absorbed (Boileau *et al.* 2002). Hence, enhanced levels of lycopene *per se* in foods may not be beneficial to human health (Tucker 2003). The absorption of lycopene is much greater from processed tomato products, such as pastes, particularly when mixed with fats. An artificial formulation in which lycopene is entrapped within whey proteins (lactolycopene) has similar bioavailability to lycopene in tomato paste (Richelle *et al.* 2002).

3.9.3 Flavonoids

A number of flavonoid compounds, more importantly the anthocyanins are efficient antioxidants. The flavonoid content was greatly enhanced through transgenic technology in tomato (Muir *et al.* 2001), soybean (Yu *et al.* 2003) and rice (Shin *et al.* 2006). While tomatoes are generally low in flovonol content, the constitutive expression of *Arabidopsis thaliana*'s AtMYB12 in tomato enhanced flavonol content greatly increasing the antioxidant potential of the transgenic tomato fruit (Pandey *et al.* 2015).

Anthocyanins are water-soluble pigments that impart red, purple, or blue colour to the plant parts that contain them. There are many dietary sources of anthocyanins, but they are either expensive and/or not readily available in developing countries. Hence, more efficient sources need to be developed. The phenolic compound content was manipulated to enhance the antioxidant capacity in transgenic potatoes (Lukaszewicz *et al.* 2004). Through conventional breeding, anthocyanin content in tomatoes could be very considerably increased in the outer skin, but not as much in the flesh. As the fruit peel is often removed during tomato processing most of the anthocyanins is lost. By boosting the levels of two transcription factors that regulate the expression of snapdragon genes involved in anthocyanin biosynthesis, anthocyanin levels were greatly enhanced in both peel and flesh of transgenic tomato fruits (Butelli *et al.* 2008). The purple fruit colour could be introgressed into other commercial cultivars of tomato. The life span of tumorogenic mice increased when fed with transgenic tomato powder at 10% of body weight (Hirschi 2009) indicating that these transgenic tomatoes can potentially contribute to a substantial increase of antioxidant content of human diets.

3.9.4 Carotenoids

Among a very large number of carotenoid compounds, about 20 commonly occur in plants performing various functions. The ripening of fruit alters the aroma, flavour, colour, and texture with direct implications for carotenoid concentrations (Barry and Giovannoni 2007). Three carotenoids, α-carotene, β-carotene and β-cryptoxanthine, are very important in human health as they are the major precursors of vitamin A (see Section 3.7.2). Many carotenoids are known to function as antioxidants in mammalian systems.

The genes involved in light signal transduction which regulates the biosynthesis and accumulation of carotenoids and other antioxidants in tomatoes have been elucidated

(Fraser *et al.* 2002, 2007). Clustered loci for accumulation of major vitamin A precursor carotenoids were identified in carrots (Santos and Simon 2002). In tomato, the levels of β-carotene (Rosati *et al.* 2000) and of carotenoids in general (Fraser *et al.* 2001), were substantially enhanced through genetic manipulation. Similarly, the levels of carotenoids (lutein in particular), were enhanced in potato tubers (Ducreux *et al.* 2005). β-carotene, expressed in the endosperm of Golden rice, is intended to ameliorate vitamin A deficiencies (Ye *et al.* 2000, Paine *et al.* 2005) (see Section 3.7.2).

3.9.5 Other Antioxidants

The chelation of minerals including radioactive heavy metals by phytic acid, is cited to project phytate as an antioxidant (see Section 3.10.2). Enhancement of the levels of other antioxidants such as vitamins C and E, β-carotene and selenium is detailed in the respective sections.

3.9.6 Thermal Stability of Antioxidants

The thermal stability of antioxidants is an important issue as most food is cooked. While there are no data on the thermal stability of antioxidants in general, Paul *et al.* (2012) showed that oryzanol, in rice bran, mustard and sunflower oils, a widely used antioxidant in foods, beverages and cosmetics, is stable even after eight hours of heating at 180°C, better in pan heating than microwave.

3.10 Mitigation of Levels of Antinutritional Factors

3.10.1 The Antinutritional Factors

Plants produce a very large number chemical compounds of diverse biochemical groups such as alkaloids, cyanogenic glycosides, flavonoids and so on, which serve as defence mechanisms to protect them from pests, pathogens and grazers. Many of these compounds interfere with metabolic processes, adversely affecting human and animal health and some may even be toxic. All such compounds are collectively recognised as antinutritional factors (ANFs). The issue of denaturing/inactivating antinutritional factors was earlier discussed by Morandini (2010) and Patil and Kameswara Rao (2015).

Most of the routine food preparation processes either denature the ANFs or even remove them from the processed foods. Nevertheless, it is considered safer to genetically reduce their levels or even prevent their production altogether in crop plants, through GE. Over the centuries scientists have bred and/or selected crop varieties with ever decreasing quantities of ANFs and increasing nutritional potential. A number of efforts were made in recent times to develop strategies, many involving GE processes, to limit ANFs in crop plants that cause malabsorption, intolerance, allergies or toxicity, as illustrated by the following examples.

3.10.2 Phytate

The principal storage form of phosphorus in plants is phytate (myo-inositol hexaphosphate), present in all plant foods. Phytate is more abundant in seed coats (hulls) of cereals and legume grains with 50% to 70% of their phosphorus in the form of phytic acid.

Phytate has some beneficial effects, but its negative impact on our health has attracted more attention. In addition to being phosphorus store, phytate is a source of cations and myo-inositol (the precursor of cell walls) and is implicated in DNA repair. The chelation of minerals by phytic acid, including radioactive heavy metals, is cited to project it as an antioxidant (see Section 3.9). The benefit of cancer prevention, particularly colon cancer, attributed to dietary fibre in whole grain and bran foods is linked to phytate, as these foods are rich in it (Patil and Kameswara Rao 2015).

Phytate chelates many divalent mineral ions, particularly iron and zinc, and forms insoluble complexes making the mineral elements unavailable to the body system even when the foods consumed are rich in them. Phytate would also affect enzyme activities that essentially need minerals like zinc. Phytate makes phosphates too unavailable. Consequently, phytate is considered a major ANF.

The enzyme phytase, more abundant in microorganisms, hydrolyzes phytic acid, facilitating the release of minerals and phosphates bound by phytate. As phytase is normally unavailable in humans and non-ruminant animals, they excrete the excess amount of phosphate polluting the environment. Phytase is often added to animal feeds to enhance the bioavailability of minerals and to reduce excretion of phosphorus.

A higher bioavailability of micronutrients, minerals and phosphorus in grains is related to the lower quantity of phytate (Stangoulis *et al.* 2007) and/or higher levels of phytase. As the protein in low phytate transgenic soybean is more easily digested than the protein in traditional soybean, transgenic plant foods are considered to be better than commercial phytase supplementation (Keshavarz 2003). Use of low phytate soybean meal along with low phytate maize significantly reduced phosphate excretion in farm birds. Low phytate grains play a role in fighting malnutrition (Raboy 2002) as the absorption of zinc (Adams *et al.* 2002) and calcium (Hambridge *et al.* 2005) was enhanced in low phytic acid maize. GE crops for low phytate and/or high phytase would result in improved human and animal nutrition. While transgenic low phytate products are fraught with regulatory problems, the USDA in 2011 has exempted the ZFN-12 low phytate maize developed using zing finger nuclease technology (see Section 3.3.5) from the regulatory oversight (Kuzma *et al.* 2016).

Transgenic soybean with fungal phytase gene showed improved phosphorus availability (Denbow *et al.* 1998). A transgenic wheat accumulated phytase from a fungal gene (Brinch-Pedersen *et al.* 2000). Lucca *et al.* (2002) obtained a 134-fold increase in thermotolerant phytase in transgenic rice with genes from the fungus *Aspergillus fumigatus*. In 2009, the Chinese company Origin Agritech got the world's first genetically engineered phytase expressing maize approved (Han 2009).

The Canadian GE '*Enviropig*' produces phytase in the salivary glands to digest phytate, releasing a lot more of phosphorus than the conventional breeds, thus reducing phosphorus pollution of the environment through excretion (Golovan *et al.* 2001). Despite regulatory approvals from the Canadian Government in February 2010, the commercialisation of this GE pig has gone on to the back burner.

The negative impact of phytate on our nutrition is real and rightly highlighted, but its extent in real time is not (Patil and Kameswara Rao 2015). Extensive literature indicates that the acidic environment of our gut as also the lactic, citric, tartaric and other acids from the diet, sprouting, fermentation and other food preparation methods and cooking practises, neutralize most of the phytate (Nagel 2010). Lactobacilli and other endogenous microflora in the gut enhance the availability of phytase.

Actually, there is more risk from phytate in raw foods and unmilled grain, which are currently projected as health promoters. In view of these factors, it would be better if sensible food preparation and cooking practises discussed by Nagel (2010) are adopted to reduce phytate and increase phytase in plant foods for human consumption (Patil and Kameswara Rao 2015), than going by the time consuming and expensive GE technological intervention.

3.10.3 Inhibitors of Digestive Enzymes

A number of commonly consumed food grains, more particularly the grain legumes, contain inhibitors of digestive enzymes (Mamatha Rao 2008). Globally, a large number of consumers of these foods suffer from severe digestive problems on account of compounds such as the Kunitz trypsin inhibitors. In order to circumvent such problems, globally available genotypes of grain legumes were screened to identify null allele varieties for the inhibitors. Two Indian genotypes of soybean (NRC 101 and NRC 102) without the alleles for the inhibitor were developed from the existing genotypes through conventional breeding and selection, and these are now ready for commercialisation (Rani and Kumar 2015).

3.10.4 Reducing Levels of Allergens

The levels of allergens were genetically reduced in the following crops:

i) **Soybean:** Silencing a major allergen in soybean (p34) through RNAi resulted in the absence of p34-specific IgE anti-bodies in the blood of sensitive individuals (Helm *et al*. 2000, Herman *et al*. 2003).

ii) **Wheat:** Gluten is a composite of gliadin and glutenin, and constitutes about 80% of the storage proteins associated with starch in wheat endosperm and some other cereals like barley and rye. Gluten imparts elasticity and other qualities to wheat dough, essential for making bread and other bakery products. Gliadin may induce celiac disease in genetically predisposed individuals or aggravate it in those already affected. Celiac disease is an autoimmune disorder affecting the digestive system, suffered by about 1% of people in general. In addition, some people may suffer from wheat allergy, which is different from celiac disease, and both can be managed by gluten-free diet. Gil-Humanes *et al*. (2010) used RNAi to shut down celiac disease-related wheat gliadin T-cell epitopes. A reduction in the levels of expression of the thioredoxin gene also reduced the intensity of allergenic response to wheat (Buchanan *et al*. 1997).

   Though only a very small fraction of the global population suffers from problems related to gluten (or zein), gluten-free products, even gluten-free beer, are already on the market. The concern about gluten allergy has led to voluntary labelling of wheat products as '*gluten free*' in the United States and some other countries. Reduction of gluten levels in wheat would adversely affect the dough quality and so the appeal of this transformed wheat is limited.

iii) **Maize:** Zein, the storage protein in maize, is also a prolamine like gluten. As zein also is suspected to be an allergen in some people, maize lines with reduced zein levels were developed through RNAi (Segal *et al*. 2003).

3.10.5 Other Significant Antinutritional Factors

Several other chemicals in plant foods may function as ANFs. As the following ANFs are more common, they were addressed for reduction of their levels in some crops: :

i) **Steroidal alkaloids:** McCue *et al.* (2003) significantly reduced levels of major steroidal glycol-alkaloids in potato using antisense constructs, while the solanine content of potato was reduced substantially through the antisense approach (Lukaszewicz *et al.* 2004), making these potatoes safer to sensitive individuals.
ii) **Cyanogenic glycosides:** Levels of cyanogenic glycosides in cassava were reduced through the expression of the cassava enzyme hydroxyl nitrile lyase in the tuberous roots (Siritunga and Sayre 2003).
iii) **Caffeine:** A number of antinutritional and other ill-effects have been attributed to excessive consumption of coffee that led to marketing **c**hemically decaffeinated coffee. However, chemical decaffeination removes several other compounds as well, affecting the flavour and taste of coffee. Preventing the expression of caffeine through RNAi removed only caffeine, leaving all other compounds and the flavour of coffee intact (Ogita *et al.* 2003).

3.11 Conclusions and Recommendations

The efficacy, safety and benefits from GE technologies in crop biofortification to ensure nutritional security have been highlighted in the preceding pages and a number of conclusions were drawn and recommendations made contextually, on diverse related issues. Zhu *et al.* (2007), Meyer *et al.* (2008), Hirschi (2009), Beyer (2010), Newell-McGloughlin (2010), Potrykus (2010), von Braun (2010), Bouis *et al.* (2011), Dubock (2013), Rawat *et al. (*2013), Kameswara Rao (2013, 2015) and Patil and Kameswara Rao (2015), among others, have emphasised the beneficial role of GE technologies could play in ensuring food and nutritional security in the developing countries. All these recommendations and the guidelines provided in the reports of the organisations on food and nutritional security, more importantly the 2016 Global Nutrition Report (IFPRI 2016), should guide policy decisions, as they focus on making smart committments to nutrition and identifying what it will take to end malnutrition in all its forms by 2030. It is hoped that the following conclusions and recommendations would also be of help in this effort:

a) Biofortified crops do not involve any additional costs of cultivation and use. Costs are involved only in the development of prototypes, farmer seed and extension programmes. To the farmers and consumers biofortified crops would cost the same as their conventional counterparts.
b) Administering single nutrients in high doses, without a clear evidence on the need to take them, has its own drawbacks such as creating a nutritional imbalance, while consuming nutrient rich foods, rather than supplements has certainly more health advantages (Nestle 2002). Almost all supplemented nutrients when taken far above the RDAs can have toxic effects (Beers 2003). There is no risk of overdosing of nutrients in food crops;

c) The current state of understanding, ability and the anticipated developments to manipulate plant secondary metabolism, and the current state of our technological competence and rapid innovation of technologies as detailed by Newell-McGloughlin (2010), Dubock (2013), Patil and Kameswara Rao (2015) and in the preceding pages, reiterate that we would certainly be able to modify the nutritional content of food and feed crops to improve human and animal nutrition and health;
d) Over a couple of dozen nutritionally enhanced crops have been developed through conventional and GE technologies, but these have remained at the proof of the concept stage, even when the technologies come free of cost to the developing countries. This situation severely discourages even taking forward the prototypes now ready, let alone developing crops with the much-needed new traits. The governments should facilitate a fast track proactive regulatory processing of these biofortified crops to benefit millions of poor needy people in the developing countries and to encourage further research on biofortification through modern technologies;
e) All countries and cultures have a valuable tradition of food crops and diets. A number of local plants are used as food ingredients that promote nutrition and sustain health. Unfortunately, acculturation led to the neglect of these foods and modern foods and diets have occupied the space. Discontinuation of use of traditional food ingredients and cooking practises, has not spelt well for the public health. Changes in life style and food habits have resulted in a number of disorders and diseases. Agricultural scientists and nutritionists should encourage people to revive cultural foods, diets and eating habits that sustained millions of people over millennia in maintaining health. Using new crops and products should be promoted in areas where conventional practises are inadequate or ineffectual. Nutritional data on local food plants, crops and products for every region in each country are very much needed, to guide local policy;
f) With a few exceptions such as golden rice, multivitamin corn and purple tomato, no biofortified food or product has a visible marker to identify the presence and quantity of the nutrient. Absence of a visual means to ascertain qualitative and quantitative content of a nutrient in fortified foods encourages the commercial processed food manufacturers to mislead the public by lofty claims. Visible markers, at least at the crop primary produce stage, such as the seed colour in flax, are needed for assurance of quality;
g) Cultivation practises adopted for nutrient enriched crops should be monitored to prevent excessive use of chemical inputs like weed, pest and disease control agents and fertilisers to, (a) protect the soil from accumulation of nitrites and nitrates, (b) protect the farmers and consumers from chemical residues and (c) to prevent interference of input chemicals with biosynthetic processes of nutrient enhancement in the crop;
h) Most research on nutrient enhancement in food crops happened in the decade around the turn of this century. Although there have been some significant developments of protocols and products during the rest of time, the progress was patchy, mainly for two reasons. One is that the enormous opposition to the new technologies and the other is heavy time and financial investment needed to develop the products using new technologies, particularly when there is no hope of their being processed through the regulatory approvals. Unless this situation changes and the hitherto developed products get into commercial production, new nutrient enriched

products are unlikely to be developed. It requires a strong political conviction to change current climate of antitech activist dominance. The silver lining to this depressing scenario is that the 2016 World Food Prize was awarded for work on biofortification to Drs. Maria Andrade, Robert Mwanga, Jan Low and Dr Howarth Bouis. This tribute to nutrient biofortification is likely to encourage development of new protocols and products;

i) There would be demands for human clinical trial data, as were made for such products as the golden rice. It would be necessary to conduct human clinical trials involving the new biofortified plant foods to determine the relative benefits of nutrient supplementation *vs* biofortification in ensuring nutritional security and to convince all stakeholders on the efficacy of modern interventions;

j) Nutritional management is a lifelong issue for the individual, but needs the cooperation of the families and facilitation by (a) the agricultural system, (b) finished food product industry, and (c) the governments. Unless the personnel who prepare food (housewives, cooks) are educated on nutritional issues, nutritionally rich foods and balanced diets, levels of compliance with dietary recommendations would be patchy, and may even cessate in course of time;

k) Identification of target traits, crops, and choice of technologies is basic to crop biofortification. This needs global thinking but local action and is prerogative of the scientific community, not to be dictated by non-scientific interests like the activist groups. The focus should be on (a) traits that benefit the maximum number of people in a large number of countries, (b) crops that are widely used as food, and (c) technologies which are fast and precise facilitating stable crop performance over years.

l) Only few crops such as wheat, rice, corn, potato, sweetpotato, tapioca, etc., which are the staple foods, are suitable for nutrient enhancement as they can provide the enhanced nutrients without additional effort or expense. Millets, the staple food of the poor in developing countries, had not been an attractive proposition for qualitative and quantitative improvement as energy source and have been of much less interest for nutritional enhancement. They should now be seriously considered for biofortification as is being done in Africa;

m) Gene stacking would facilitate delivery of several traits in the produce of a single crop and so a single food ingredient, a good example being the five gene stacked three vitamin corn (Naqvi *et al.* 2009). The anticipated socio-economic benefits of multi-biofortified rice in China were already highlighted by De Stuer *et al.* (2012);

n) Certain basic socio-economic criteria have to be met to ensure food and nutritional security for the poor in the developing countries (Kameswara Rao 2013, 2015, Patil and Kameswara Rao 2015). These are:
   i) *Availability* of sufficient quantities of appropriate quality foods on a continued basis. It is the responsibility of the scientific establishments to design interventions and technologies and develop crop varieties appropriate to diverse needs;
   ii) *Accessibility* is having appropriate diverse foods for a nutritious diet in sufficient quantities, within reach of all sections of the population. This is basically the responsibility of the governments and the marketing system. An impressive range of raw and processed foods marketed by the private sector is available even in the developing countries, but largely inaccessible to the semi-urban and rural populations;

iii) *Affordability* is having sufficient financial resources to purchase what is available, accessible and required. The majority of poor in the developing countries cannot afford even the basic food needs. It is for the governments to ensure that the required quality and quantity of nutritious food reaches them, through food security programmes. Unfortunately such programmes are usually focused on carbohydrate rooted calorie intake, and neglect even protein and fat components of food security. So far micronutrient security has not gained the active attention of planners; and

iv) *Knowledge* of basic nutrition and care. Tradition in most countries is rich in health care wisdom, but the contemporary populations mostly lost track of the body of knowledge of balanced diets. The tragedy is that even educated people who can afford diverse nutritious foods suffer from nutritional deficiency disorders, while the encapsulated nutritional supplements they avidly consume largely fail them (Kameswara Rao 2013). In this context the government should put in place robust awareness programmes, devoid of corporate influences and advice from half-baked physicians and nutritionists;

o) It is near impossible to estimate the quantity of different nutrients consumed by individuals. Even when an adequate quantity of nutritious food was consumed, two biological constraints restrict us from deriving the full benefits (Kameswara Rao 2005, 2013, Patil and Kameswara Rao 2015):

i) *Bioavailability* is about the quantity of the nutrient that becomes available to the body system. Even under the best of circumstances, the entire quantity of a nutrient consumed may not become available to the body system, due to certain chemical compounds (phytate and some mineral elements) preventing its release; and

ii) *Bioconversion* is related to the quantity of a nutrient available to the body system (such as β-carotene released) against the quantity of the conversion product (such as vitamin A). A number of factors, including the state of health, which vary from individual to individual, influence bioconversion (Kameswara Rao 2005). While certain food components promote bioconversion (vitamin C, carotenoids and iron), certain others (antinutritional factors) retard the process (Kameswara Rao 2005). All that is ingested is not released nor utilised, as both bioavailability and bioconversion vary from food source to food source and are also dependent upon the kinds of foods and drugs we take from time to time;

p) The farmers would not adopt any crop that involves high seed or cultivation costs or crops that need a change in the familiar cultivation practises. The governments should facilitate a cost free transfer of technology as in the case of golden rice. When this is not possible, the governments should provide the high tech seeds at subsidised costs or even free of costs, to the farmers;

q) A large number of consumers cannot afford or may be unwilling to pay the high costs of the new enriched products. The governments, especially of the developing countries, should ensure that the new products are priced the same as their conventional alternatives or subsidise or even supply at zero costs to the consumers;

r) As most genes express throughout the plant body, after the targeted nutritionally fortified edible parts like fruits, seeds, tubers and so on, are harvested, the nutrients in the inedible parts should be extracted and added to processed foods. They can also be administered as encapsulated supplements. Widely used food products such as those of tomato, corn, soybean and so on, should be made from biofortified varieties of these crops;

s) Prevention of wastage of fortified crop produce and food products is very important as over a third of the food produced, more than 1 billion tons of edible food, is wasted every year; and
t) Public education to enhance awareness of (i) the importance of nutrients and their intake, (ii) common sources of nutrients, (iii) methods of fortification, and (iv) diets that include not only conventional foods but also GE crops and products that improve nutrient intake, is very crucial in ensuring nutritional security. Public education programmes should be conducted through all visual, electronic and print media in local languages so that they reach the uneducated even in remote corners of the developing countries. Public education will be more effectively done by focusing on the school and pre-degree children, a channel now widely exploited by prepared and supplemented food manufacturers. Children would influence the previous generation, make their parents buy new products and carry the information and knowledge to the next generation. Food, diet, nutrition and health should be a part of general education up to the pre-degree levels. To enhance public confidence in the information disseminated and products promoted, the initiative should come from the public sector institutions.

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