Crop Biofortification Through Genetic Engineering : Present Status and Future Directions

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ABSTRACT

Global food system is failing to deliver adequate quantities of healthy, nutritionally balanced food, especially to the resource-poor underprivileged people leading to micronutrient malnutrition. The malnutrition of minerals (Fe, Zn) and vitamin A are major food-related primary health problem among populations of the developing world including India where there is a heavy dependence on cereal-based diets and limited access to meat, fruits and vegetables. Vitamin A deficiency (VAD) alone is significant from the public health point of view resulting in over 330,000 child deaths every year, and about 57% of preschoolers and their mothers having subclinical VAD leading to increased morbidity and risk of mortality. While therapeutic supplementation of vitamin A is currently being addressed through sponsored nutrition programmes, they are not sufficient in covering the affected populations. Biofortification of important crop plants through biotechnological applications is a cost-effective and sustainable solution for alleviating VAD. Genetic engineering is the obvious alternative to enhance the β-carotene levels in crop plants. The development of the 'golden rice' proved that, it is possible to redirect a complete biosynthetic pathway of carotenoids by genetic engineering of multiple genes encoding key enzymes of the pathway. Recently, there have been several reports on the development of transgenic crops for enhanced levels provitamin A content in crops like maize, tomato, cassava, potato and mustered. At ICRISAT, transgenic events of groundnut and pigeonpea carrying either a single maize phytoene synthase 1 (psy1) gene or both psy1 and tomato β-lycopene cyclase (β-lyc) have been developed through Agrobacterium-mediated genetic transformation. Preliminary results showed a significant increase in the total carotenoids and β-carotene levels in the transgenic events. Provitamin A enrichment of these crops could have a significant impact on the nourishment and nutrient interactions by playing a major role in the bioavailability and metabolic efficiency in the affected populations.

Key Words: Vitamin A, biofortification. nutritionally balanced food, genetic transformation, malnutrition

INTRODUCTION

Over a billion people in the developing countries suffer from an insidious hunger known as micronutrient malnutrition. It is caused by poor quality diets, characterized by high intakes of staple crop food but low consumption of animals and fish products, fruits, legumes, and vegetables which are rich sources of bioavailable minerals and vitamins. Even mild levels of micronutrient

malnutrition may damage cognitive development and lower disease resistance in children. Despite intensive efforts to reduce micronutrient malnutrition, more than 40% of the world's population is suffering from at least one of the major micronutrient deficiency, i.e., iron, zinc, vitamin A or iodine (FAO 1997; WHO 1998; Mason et al 2001) and their prevalence is rising, especially in developing and undeveloped countries. Estimates suggest that some 815 million worldwide suffer from micronutrient deficiency (Underwood 2003). Sufficient micronutrients, minerals and vitamins in the daily diet are one of the prerequisites for human health (Caballero 2003; Black 2003). During the 'Green Revolution' towards food security through increasing the yield of staple crops, little thought was given to human health and the nutritional value of diets. Depleted soils, rudimentary farming and agricultural techniques that characterize farming practices in most developing countries have resulted in food crops that are low in required micronutrients and vitamins and subsequently diseases associated with these deficiencies. Numerous publications have demonstrated the negative impacts of the diet-related micronutrients deficiencies such as mental retardation, impaired eye sight, diabetes, hypertension and increased susceptibility to infectious disease (Lynch and Green 2001; Beard 2001; Shankar and Prasad 1998; Gilbert and Foster 2001). These are, as a result of compromised immune function due to inadequate sources of nutritious and quality foods. Deficiencies of iron, zinc, vitamin A and iodine not only compromise the immune system, but also can irreversibly retard brain development.

The undeveloped countries, especially in the Sub- Saharan Africa have higher mortality rates due to infectious disease. It has been estimated that vitamin A deficiency may contribute to as much as 23% of the child mortality in these countries (Mason and Gracia 1993). Micronutrient deficiencies such as those due to iron, zinc, iodine and others are highly prevalent (Brown et al 1998; Haddad et al 1999; Bloem and Darnton-Hill 2001). The numbers of people known to be affected world-wide by iron deficiency is over three billion, iodine deficiency is over two billion and vitamin A deficiency accounts for over 230 million children (Graham et al 2000). There is an urgent need for better quality foods with adequate micronutrient levels. Cereals and legumes are the most important staple crops that are important source of vitamins, minerals and essential amino acids that can be biofortified to combat micronutrient malnutrition.

Biofortification is the process of breeding food crops that are rich in bioavailable micronutrients. These crops load high levels of minerals and vitamins in their seeds and roots, which are then harvested and consumed. Biotechnological tools have opened up the possibilities to introduce genes responsible for the biosynthesis of micronutrients into staple foods, such as vitamin A in rice, and to enhance the efficacy of traditional breeding approaches. Gene mapping has enabled the identification of those varieties within a crop that have high micronutrient content and the relevant genes. This then, can speed-up the conventional breeding process to produce agronomically viable micronutrient-dense crop varieties. In contrast to plant breeding, the techniques of genetic engineering allow the transfer of heritable traits between completely unrelated species. In recent years, genetic engineering methods have been applied for improving the nutritional quality of the staple crops.

MICRONUTRIENT MALNUTRITION ASSOCIATED HEALTH PROBLEMS

Zinc

Zinc is an essential component of over 300 enzymes needed by the body for metabolism (WHO 2002). Zinc deficiency leads to dwarfism and hypogonadium and pregnancy-related complications, and even death. After birth, it leads to improper development of child and reduced cognitive abilities. In adults, it adversely affects the ability to work and longevity. Recommended dietary allowance (RDA) values differ among age and sex group for each and every nutrient, The RDA value for zinc is 10 mg for children, 15 mg for men and 12-15 mg for females (Anonymous 2001).

Iron

Anaemia is the most common consequence of severe iron deficiency that occurs as a result of reduction in the oxygen carrying capacity of red blood cells. Iron deficiency symptoms are more prominently visible during pregnancy due to high iron requirement, periods of growth and development or when iron is lost because of parasitic infections such as hookworms. During pregnancy, deficiency of iron can result in serious consequences for both mother and the baby. Iron deficiency anaemic (IDA) mothers have higher risk of mortality during childbirth, and an increased incidence of low-birth weight babies (Dallman et al 1980; Milman and Kirchoff 1992). The deleterious effects of anemia in infants, preschool children and school going children include reduced cognitive capabilities, mental performance and improper physical growth (Vyas 1984). IDA diminishes the stamina and work capacity of adults by 10-15 %. RDA for iron is 10 mg for children, 12-18 mg for men and 15-18 mg for women (Anonymous 2001).

Zinc and Iron Interactions

Most of the work for combating iron and zinc micronutrient deficiencies has been done in combination since their interactions are mineral-mineral type among chemically similar members of the transition metal series, which have been well characterized in plants and animals (Hill and Matrone 1970). While both synergistic and antagonistic interactions can occur, the competition between Fe^{2+} and Zn^{2+} for the bond with plasma transferrin is an antagonistic interaction that is well documented (Georgievskii et al 1982). However, this effect is not as likely to be significant if the subject is deficient in these nutrients and less probable when iron and zinc are given as food rather than as soluble supplements. Such synergistic effect strongly indicates that breeding for staple crops that are dense in both iron and zinc is required in order to effectively address iron-zinc deficiency.

Vitamin A

Since vitamin A is essential for clear vision and cell differentiation, its deficiency results in night blindness and ultimately blindness if not treated properly. Growth retardation and damage of mucous membrane of the digestive and respiratory tracts is the other disorders due to vitamin A deficiency (VAD). Children with VAD are likely to be anemic, have impaired growth and be at increased risk of severe morbidity from common childhood infections such as diarrhea diseases and measles. VAD affects 100-400 million children worldwide and about 20,000-50,000 preschool children become blind every year. Many developing countries depend on plant foods to meet their vitamin A requirements. Daily per capita availability of vitamin A has been estimated to 600 -1500 μg in adults (Anonymous 2001).

Vitamin C

Vitamin C or ascorbic acid is a water-soluble vitamin and an essential component in human nutrition that is required for normal functioning of the body. It improves cardiovascular and immune cell functions, and is used to regenerate α-tocopherol (vitamin E). vitamin C, therefore, must be obtained from dietary sources, as it cannot be stored in the body. It plays an important role in all physiological and metabolic processes. Ascorbic acid is crucial to the maintenance of a healthy immune system and is required for synthesis of collagen, carnitine and neurotransmitters. As a consequence, its most vital role in human body is as water-soluble antioxidants. RDA value is 40-45 mg for children and 45-60 mg for men and women (Anonymous 2000).

Vitamin E

Vitamin E is a fat-soluble vitamin, which protects vitamin A and essential fatty acids from oxidation in the body cells and prevents breakdown of body tissues. It exists in eight different forms, i.e., four tocopherols (alpha-, beta-, gamma- and delta-), and four tocotrienols (also alpha-, beta-, gamma- and delta-) where each form has its own biological activity. Alpha-tocopherol (α-tocopherol) is the most active form of vitamin E in humans. They are the primary form of vitamin E in the seed endosperm of most monocots, including agronomically important cereal grains such as wheat, rice, and barley. They are also found in the seed endosperm of a limited number of dicots,

including Apiaceae species and certain Solanaeceae species such as tobacco (Kamal-Eldin and Appelqvist 1996). Oilseeds are particularly rich in tocochromanols with an average concentration of 10-fold higher than other plant tissues (Karunanandaa et al 2005). Vitamin E deficiency results mainly in neurological symptoms, including impaired balance and coordination (ataxia), injury to the sensory nerves (peripheral neuropathy), muscle weakness (myopathy), and damage to the retina of the eye (pigmented retinopathy). RDA value is 8-15 mg for men and women (Anonymous 2000).

STRATEGIES FOR FORTIFICATION

There are several ways of combating nutrient deficiencies in developing and undeveloped countries that include dietary diversification, food fortification, supplementation, agronomic practices and crop impromenent in various ways as follows:

Dietary Diversification

Dietary diversification is undoubtedly the most logical and sustainable strategy to improve micronutrient malnutrition. It involves the attempt to increase the consumption of grain, vegetables and suitable fresh fruits. But this approach is more complex, involving a number of factors including accessibility, affordability, bioavailability and change in dietary habits.

Food Fortification

Fortification is defined as the addition of one or more essential nutrients to food, for correcting the deficiency in the population or specific population groups (FAO 1996). Fortification of food is routine and has proven to be very efficient for certain micronutrients; for example, the iodination of salt and flour and fortification with iron and vitamins in sugar (Darnton-Hill and Nalubola 2002). Wheat products are fortified with various micronutrients such as zinc, iron, riboflavin, thiamine and niacin to increase the nutritive value of the food. It is achieved through the addition of supplements to food in the best form for a particular type of food so that the food consistency, taste, appearance, etc. are not altered. Fortification is, however, different for each micronutrient, and is particularly difficult for iron owing to its rapid oxidation (Boccolo and Iyenger 2002). Compounds that are less sensitive to oxidation than iron are easier to handle in food fortification. Micronutrients fortification during food processing is difficult and most of the micronutrients are lost during processing for food or feed (Cheng and Hardy 2003). Efficacy trials are also needed to determine the actual capacity for correcting nutritional deficits in a target population which are much more expensive and difficult to conduct on the other side; fortified foods are more expensive than standard products, and therefore, potentially less affordable by those at the greatest nutritional risk.

Supplementation

It refers to a technical approach in which nutrients are added directly by means of syrup or pills to make up for the deficiencies in food. Supplementation is most appropriate for target population with a high risk of deficiency or under special circumstances, such as during pregnancy or in an acute food shortage. It can be effective and economically viable way of alleviating deficiencies but it has failed due to a lack of adequate infrastructure and education in the developing countries. This is however expensive and not a feasible option in poorer countries.

Under normal conditions, supplementation programmes are used only as a short-term measure and are then replaced with long term, sustainable food based measures, such as fortification and dietary diversification. Supplementation is thus easier for compounds that body can store such as vitamin A than with micronutrients that need to be distributed at much higher frequency. This approach, is useful for producing a rapid improvement in iron status in anemic individuals, but is also expensive and usually has poor compliance because of the unpleasant side effects of medicinal iron. In India, although the vitamin A supplementation program has been in operation since early 1970 **(**Reddy 2002**),** the national coverage of all the children has been hard to sustain over time.

Agronomical Practices

Alternatively, the micronutrient content of staple food crops can be increased by agronomic practices. Nutrient management in the field is of high ecological and economic importance; however, it is currently practical for only some nutrients such as, zinc, selenium and iodine deficiency, which rises when nitrogen level increases in soil. It is not very effective with other minerals, such as iron, because of its limited mobility in the phloem and rapid oxidation in soil and the success rate varies according to the geographical location. Some micronutrients can also be delivered through irrigation water, but the toxic nature of these elements will create negative impact on the environment. An alternative approach to fortification through agricultural management and food processing is the accumulation of micronutrients directly in cereal seeds using conventional breeding or targeted genetic engineering (Zimmermman and Hurrel 2002).

Conventional Breeding

Biofortification is an option with potential for applications not only in the developing countries, but also in the developed countries. It refers to crop breeding for varieties with higher micronutrient content. Exploiting the genetic variation in crop plants for micronutrient density is one of the most powerful tools in biofortification of crops, which can reach the poor in rural areas, with low recurrent costs and long-term sustainability. Breeding for specific nutritional qualities require nutrient density traits in high yield cultivars and strategies based on these genetic findings to determine the best selection technique. Micronutrient-enrichment traits are available within the genomes of some major staple food crops, which can be used for substantial increases in the levels of iron, zinc and pro-vitamin A carotenoids as well as other nutrients and health-promoting factors without negatively impacting crop yield. The reduction of anti-nutrient substances that inhibit micronutrient bioavailability or the increase in substances that promote micronutrient bioavailability from staple plant foods are both options that could be pursued in breeding programs. However, care needs to be taken not to compromise agronomic performance and sufficient attention paid to possible beneficial roles of compounds that reduce the bioavailability of trace minerals.

The use of biotechnological tools, such as molecular marker-assisted selection, significantly increases the pace and prospects of success for breeding to improve the nutritional value of staple food crops. The highest micronutrient densities, which are approximately double that of popular modern cultivars and indicating the existing genetic potential, can be successfully combined with high yield. Major success stories in this field are QPM maize (Quality Protein Maize), high carotene sweet potato and maize (Iglesias et al 1997; Harjes et al 2008). Unlike mineral micronutrients, vitamin A is not obtainable from plants; however, its precursors, the pro-vitamin A (ß-carotene) are found in high levels in yellow maize, sorghum and pasta wheat, higher still in cassava and sweet potato but at low levels in other staples such as wheat, rice and groundnut. Breeding has its own limitations in some crops due to a narrow range of the germplasm, lack of micronutrients traits in wild species, and hybridization barriers. It is not possible in vegetatively propagated crop plants. Genetic engineering can overcome these problems and it may be economical for the poor sections of society.

Biotechnological Approaches

Biotechnology and genetic modification techniques are being optimized for the production and development of healthy foods and improvement in the levels and activity of biologically active components in food plants (phytochemicals). Genetic engineering techniques have mostly been targeted at increasing yields of cash crops in the developing countries. However, the crops with improved food quality have gathered much less attention. Genetic modifications in plants include mutation breeding, improved conventional breeding, molecular breeding, transgenic breeding and somatic hybridization, (Christou 1997; Mackay

1991; Mazur 2001; Yan and Kerr 2002; Bouis 2003). Gene marking and engineering techniques allow identifying the specific plant gene or genetic material that control nutrient contents. Such material is selected and used for developing varieties with higher micronutrient contents. Furthermore, it provides insight into the potential for application of transgenic technology in developing improved quality and functional foods for human nutrition and health. The production of increased levels of β-carotene (the precursor to vitamin A) in plants is especially important, as its precursor, lycopene has been shown to have physiological chemo-preventive effects with regard to various cancers (Yan and Kerr 2002). Furthermore, lycopene, commonly found in various carotenoid-containing plants such as tomatoes and carrots, is an essential ingredient in maintaining eye health and vision.

Biotechnology enables the selection of successful genotypes, isolation and cloning of favorable traits and the creation of transgenic crops for sustainable agriculture (Sharma and Oritz 2000). The advent of biotechnological tools including marker-assisted selection and gene transfer across the species barrier has opened up novel opportunities for enhancing the seed-quality, disease and pest resistance, viral resistance, abiotic stress tolerance (Sharma and Anjaiah 2000). The application of plant biotechnology to improve the nutritional content of staple food crops has perhaps the greatest potential to benefit global health. Genetic engineering methods can be used to increase the trace element content of staple foods such as cereals and legumes, which can be achieved by insertion of genes with the ability to produce the desired nutrients that are typically deficient. It may involve the identification and insertion from another source, or deletion of a gene to improve the desired trait like micronutrient density. This may be achieved by the introduction of genes that code for trace element-binding proteins, over expression of storage proteins already present or the expression of other proteins that are responsible for trace element uptake into plants.

BIOFORTIFICATION THROUGH GENETIC ENGINEERING

In contrast to plant breeding, the techniques of genetic engineering allow the transfer of heritable traits between completely unrelated species. In recent years, genetic engineering techniques have been used to introduce new traits into commercially important plants thereby producing combinations of features which could not been achieved by traditional breeding. Several key factors play an important role for successful genetic transformation of crop plants including the development of reliable tissue culture and regeneration methods, preparation of gene constructs with suitable promoters, efficient transformation techniques, recovery and multiplication of transgenic plants, characterization of transgenic plants for the introduced traits and transfer of transgenes into elite cultivars by conventional plant breeding methods (Sharma et al 2005). Genetic engineering methods have been used for enhancement of micronutrients in staple crops. These are a few examples on micronutrient enrichment in staple crops through transgenic approaches

Iron

The introduced the *Naat-A* (nicotianamine synthase) gene with a 35S promoter in rice was shown to result into some iron-efficient rice strains in calcareous soil (Takahashi 2003). Nicotianamine synthase stimulates the production of siderophores in the roots that improves the uptake of iron in the plants. Overexpression of zinc transporter protein from *Arabidopsis* with the ubiquitin promoter has been reported to increase the uptake and transport of zinc and iron in transgenic barley plants (Ramesh et al 2004). Goto et al (1999) reported a two to three-fold increase in iron in transgenic rice expressing the soybean ferritin gene. The transgenic tobacco plants expressing the same gene under influence of a constitutive promoter showed approximately 30% more iron in the leaves than that of non-transgenic leaves (Goto et al 2000). To increase iron content in rice seeds, the ferritin gene from *Phaseolus vulgaris* was expressed in rice endosperm under the control of the glutelin

promoter that resulted in over two-fold increase in the iron content (Lucca et al 2002). Similarly, Vasconcelos et al (2003) expressed the ferritin gene again under the control of the endosperm-specific glutelin promoter and demonstrated an increase of Fe and Zn content not only in the whole grain, but also in the polished grains. Liu et al (2004), observed the iron content in the milled transgenic rice with ferritin gene from soybean under rice glutein promoter to be up to 64% higher than that of the untransformed rice. Anai et al (2003), constructed a chimeric gene consisting of a maize *Ubi1-P-int* and a soybean *GmFAD3* cDNA and introduced into rice plants and found that alpha-linolenic acid content of the transgenic seeds increased dramatically up to ten-folds than that of the control, and transgene was stably inherited in the next progenies. The expression of recombinant humanlactoferritine (rHLF) in rice endosperm produced not only 5 g rHLF per kg dehusked rice grains, but also increased by about two-folds Fe content (Nandi et al 2002).

The most important way to improve the iron content in crops is through the enhancement of the absorption, transport and accumulation of iron. The absorption rate of iron in plant food source is lower than 10%. For example it is, 1% in rice, 3% in corn and black bean, 4% in lettuce, and 5% in wheat (Cheng and Hardy 2003). This is due to the fact that many inhibitory factors in plant food source impair the absorption of iron. Such factors include phytic acid, oxalic acid, and carbonate, which form an insoluble salt with iron. Certain amino acids (e.g., cysteine) and proteins also have an important role in determining the availability and uptake of Fe during digestion. A cysteine-rich metallothionein-like protein has been expressed in rice endosperm, resulting in a nearly seven-fold increase in cysteine fraction content of the seed, which had a positive impact on Fe uptake. Iron absorption is influenced by the presence of phytates and tannins in grains such as maize. Modified maize in which phytates have been reduced by insertion of the phytase gene resulted in greater availability of iron, despite no change in overall content of iron (Raboy 1996; Mendoza et al 1998; Bouis 2003). The increased iron absorption from low-phytic acid maize is particularly applicable in areas where maize and its products are staple foods. This also applies to other grain cereals where iron absorption is compromised by the presence of phytates and other anti-nutritional components. Transgenic wheat a expressing a high level of phytase has been developed with *Aspergillus niger* phytase gene for obtaining higher availability of iron and zinc (Brinch-Pedersen et al 2000) and rice (Lucca et al 2001a,b). Samuelsen et al (1998) showed that the transgenic tobacco plant expressing a yeast ferric reductase gene increased leaf iron content by 50%. Similarly, increasing the concentration of the storage proteins like phytoferritin and metallothionein could increase the content of Fe and Zn absorption, respectively.

Vitamin A

Provitamin A or β–carotene is one of plant carotenoids, a major precursor for vitamin A which is generally thought to be the most important for humans. In addition to being a precursor for vitamin A, β-carotene is an important antioxidant that helps to prevent harmful free radical damage in the body. Dietary carotene has about half of the biological activity of vitamin A because of the low efficiency of carotene to retinol conversion. Vitamin A deficiency is more widespread in parts of countries where rice or wheat or cassava is the staple food when compared to areas where the major staple is yellow maize, millet or sweet potato, all of which provide considerable amounts of pro-vitamin A. In tomato, seven major biosynthetic steps, and more than 20 genes, have been well characterized in the synthesis of carotenoids. Hauge and Trost (1928) described a major gene for carotene content in maize, and designated it the Y (yellow) locus that is incompletely dominant. The enormous progress made in the cloning of β-carotene genes has opened up the possibility of modifying and engineering the carotenoid biosynthetic pathway in plants, especially in food crops, considering the importance of carotenoids like β-carotene in human nutrition and health. Several approaches have been used to increase the level of β-carotene in some key crop plants:

Rice

Engineering β-carotene biosynthetic pathway into the rice endosperm was novel in the sense that it was aimed at a tissue that was totally devoid of the pathway. Carotenoids do not accumulate in the rice endosperm; however, the general precursor geranylgeranyl pyrophosphate (GGPP) is present in this tissue. Burkhardt et al (1997), for the first time demonstrated that it is possible to engineer β-carotene biosynthetic pathway in a non-photosynthetic, carotenoid-lacking plant tissue by transforming a *japonica* rice variety T309 with daffodil phytoene synthase gene driven by a seed-specific promoter from glutenin (Gt1), where the transgenic plants accumulated phytoene in the endosperm. This result was extended to the ultimate β-carotene accumulation by Ye et al (2000), who introduced three genes, a daffodil phytoene synthase (*psy*1) gene under the control of seed specific Gt1 promoter, an phytoene desaturase gene (*crt*I) from *Erwinia uredovera* (codes for enzymes mediates four desaturation steps, for which two plants enzymes are required) driven by CaMV 35S promoter, and daffodil lycopene β-cyclase (*lcy*) driven by CaMV 35S promoter into T309 *japonica* rice line. This resulted in the accumulation of carotenoids (surprisingly, mostly β-carotene) in the endosperm. Interestingly, they were also able to produce β-carotene only by introducing the phytoene synthase and phytoene desaturase activities in the absence of heterologous β-cyclase. This work was further extended to several widely grown indica rice varieties from different eco-geographical regions of Asia (Datta et al 2003). Recently, it has been observed that source of the phytoene synthase gene plays an important role in alleviating β-carotene level and thought to be the rate limiting and major regulatory step for carotenoid biosynthesis. Paine et al (2005), developed golden rice 2 by introducing the maize *psy*1 gene in combination with the *crt*I (carotene desaturase) gene and observed an increase of up to 23-folds of total ß-carotene when compare to the golden rice 1.

Tomato

Rosati et al (2000) were able to enhance the conversion of β-carotene from lycopene, which is normaly present in high amounts in tomato fruit, by transforming with β-*lcy* gene from *Arabidopsis* driven by fruit-specific promoter. They also reported an increase in the total carotenoid level in the fruit. In another study, while the expression of *crtI* gene driven by CaMV 35S promoter was shown to increase the lycopene content of transgenic tomato fruits, unexpectedly this also resulted in 50% decrease in total carotenoids, mainly at the expense of lycopene while β-carotene increased by about three folds (Romer et al 2000). Fray et al (1995) reported overexpression of tomato fruit phytoene synthase by transforming phytoene synthase cDNA (*psy*1) under the control of CaMV 35S promoter in tomato, which led to a reduction in the levels of gibberellin A_1 (GA_1) and chlorophyll. This result highlighted the complexities of manipulating carotenogenic pathway, as the increased flux towards the direction of one product may affect the other essential metabolites, thereby affecting the phenotype. However, Fraser et al (2002), transformed tomato with phytoene synthase (*crt*B) gene from *E. uredovora* driven by fruit-specific tomato polygalacturonase promoter and reported a 2-4 fold higher carotenoid levels in the primary transformants, whereas, phytoene, lycopene, β-carotene and lutein levels also increased by two-folds. This suggests that manipulating carotenogenic pathway in a seed-specific, rather than constitutive manner could be the right option. There are also reports of transgenic tomatoes containing carotenoids that are not normally present in the fruit. Zeaxanthin and β-cryptoxanthin containing fruits have been produced through the expression of two cDNAs: the *Arabidopsis* β-*Lcy* and *Capsicum* β-carotene hydroxylase (β-*Chy*), both with the tomato Pds promoter (Dharmapuri et al 2002).

Maize: The generation of transgenic maize with enhanced pro-vitamin A content in their kernels due to the overexpression of the bacterial genes *crtB* (phytoene synthase) and *crtI* (phytoene desaturase) under the control of a 'super g-zein promoter' for endosperm-specific expression was reported by Aluru et al (2008). Data showed an increase of total carotenoids of up to 34-folds with

a preferential accumulation of β-carotene in the maize endosperm. Zhu et al (2008) reported combinatorial nuclear transformation - a novel method for the rapid production of multiplex-transgenic plants and for modifying a complex metabolic pathway in maize. They introduced five carotenogenic genes controlled by different endosperm-specific promoters into a white maize variety deficient for endosperm carotenoid synthesis. Distinct metabolic phenotypes were observed, which also allowed the identification of complementing rate-limiting steps in the pathway. This process allowed the generation of plants with extraordinary levels of β-carotene and other carotenoids, including complex mixtures of hydroxyl carotenoids and keto-carotenoids. Combinatorial transformation is a versatile approach that could be used to modify any metabolic pathway and pathways controlling other biochemical, physiological, or developmental processes. Naqvi et al (2009) transformed elite inbred South African transgenic corn plants in which the levels of vitamins were shown to increase specifically in the endosperm through the simultaneous modification of 3 separate metabolic pathways. The transgenic kernels contained 169-folds the normal amount of β-carotene, 6-folds the normal amount of ascorbate, and double the normal amount of folate where the trait was found to be stable at least through to the T_3 homozygous generation.

Mustard

The introduction of the *crt*B (phytoene synthase) gene from *E. uredovora* in *Brassica napus* under the control of seed-specific napin promoter was shown to result in a 50-folds increase in the total carotenoid level (Shewmaker et al 1999). Surprisingly, the predominant compounds accumulating in the seeds were α- and β-carotene, and not lutein, which is the predominant carotenoid in non-transformed control seed. While sterol levels remained same, tocopherol and chlorophyll levels were significantly reduced in the transgenic seed. Ravanello et al (2003), tested three gene construct carrying the additional bacterial genes for the enzymes geranylgeranyl diphosphate synthase (*crt*E), phytoene desaturase (*crt*I) and lycopene β-cyclase (*crt*Y) engineered in conjunction with phytoene synthase (*crt*B) in transgenic canola seed. The transgenic seeds from two genes construct including bacterial *crt*B and the plant lycopene β-cyclase showed an increase in the levels of total carotenoid which was similar to that previously observed by expressing *crt*B alone but minimal effects were observed with respect to the ratio of β- to & carotene compared to the original construct. However, the β - to α-carotene ratio increased from 2:1 to 3:1 when a three-gene construct consisting of the bacterial phytoene synthase, phytoene desaturase and lycopene cyclase genes expressed together. This result suggests that the bacterial genes may form an aggregate complex that allows in vivo activity of all three proteins through substrate channeling. It allows further manipulation of the carotenoid biosynthetic pathway for downstream products with enhanced agronomic, animal feed and human nutritional values. In 2008, Yu et al used RNAi approach for carotenoid enhancement by downregulating the lycopene epsilon cyclise gene and found increased levels of β-carotene, zeaxanthin, violaxanthin and, unexpectedly, lutein.

Potato

Potato tubers contain low levels of carotenoids, composed mainly of the xanthophylls, lutein, antheraxanthin, violaxanthin, and xanthophyll esters. However, none of these carotenoids have provitamin A activity. Romer et al (2002), observed dramatic increase in the zeaxanthin content and the total tuber carotenoid content up to 5.7-folds due to the down-regulation of zeaxanthin epoxidase in the tubers. Similarly, Diretto et al (2006), silenced the lycopene ε-cyclase (ε-LCY) by *Agrobacterium*-mediated transformation of an antisense fragment of this gene under the control of the patatin promoter. The transgenic tubers thus produced showed a significant increase in β-carotenoid levels where the β-carotene showed a maximum increase of about 14-folds while the total carotenoids increased up to 2.5-folds. Interestingly, these changes were not accompanied by a decrease in lutein, thereby suggesting that ε-LCY is not rate limiting for lutein accumulation. Subsequently, the non-heme β-carotene

hydroxylases *CHY1* and *CHY2* in the tuber were also silenced where CHY silenced tubers showed more dramatic changes in carotenoid content than ε-LCY silenced tubers, with β-carotene increasing up to 38-folds and total carotenoids up to 4.5-folds. These changes were accompanied by a decrease in the immediate product of β-carotene hydroxylation, zeaxanthin, but not of the downstream xanthophylls, violaxanthin and neoxanthin. Together with ε-cyclization of lycopene, β-carotene hydroxylation is another regulatory step in potato tuber carotenogenesis (Diretto et al 2006).

Metabolic engineering of plant carotenoids has been achieved through different strategies such as "push strategies", in which a gene encoding a rate-limiting step in the pathway is overexpressed, and "block strategies", relying on the silencing of a biosynthetic step situated immediately downstream of the compound whose levels are to be increased. Transgenic potato plants have been produced by expressing *Erwinia uredovora crtB* gene encoding phytoene synthase under the control of tuber-specific promoter (Ducreux et al 2005). In these tubers, violaxanthin, lutein, antheraxanthin, and β-carotene were found as major carotenoids. Diretto et al (2006), transformed potato with three genes of bacterial mini pathway, phytoene synthase (*crtB*), phytoene desaturase (*crtI*) and lycopene β-cyclase (*crtY*) from *Erwinia*, under tuber specific or constitutive promoter. Expression of all three genes, under tuber specific promoter, resulted in tubers with a deep yellow (''golden'') phenotype without any adverse leaf phenotypes.

Groundnut

Legumes provide an excellent combination of nutrients for a balanced human diet. They alone contribute 33 % of the dietary protein needs of humans (Graham and Vance 2003). For most of the legumes, the major carotenoids detected include β-carotene, lutein and cryptoxanthin. Lutein is found in most of the legumes and is clearly the major carotenoid, followed by other carotenoids like β-carotene and cryptoxanthin. Among the legumes, lutein can make up for over 60% of the sum of the total carotenoids, whereas β-carotene and cryptoxanthin were detected in (<20%) low proportions (Siong et al 1995). In the case of groundnut, maximum concentration of carotenoid occurs in the immature kernels and diminishes as the seeds advances to maturity (Pattee et al 1969). Since plant breeders have not yet found any wild and mutant lines with high β-carotene content in the groundnut to be used in breeding, genetic engineering may be the only approach to enhance β-carotene in this crop.

Keeping in the view the limitations of breeding to produce high β-carotene groundnut, studies have been carried out at ICRISAT to enhance its provitamin A of the β-carotene content to combat malnutrition through genetic transformation (Sharma and Anjaiah 2000). Preliminary results indicated 100-folds increase in β-carotene content as compared to the untransformed plants in the case of groundnut. In-house cloning was carried out for the amplification of the *phytoene synthase* gene from cDNA of the maize (Zm *psy1)* and it was fused with constitutive promoter or the oil body-specific oleosin promoters (Sharma KK unpublished results). Following molecular characterization, and on the basis of total carotenoids and HPLC data, 14 transgenic events were selected and advanced to T_2 generation. The β-carotene levels ranged from 0.02-0.72 μg/gm in T_1 seeds which is a decent 70-folds increase than the untransformed control plants. Molecular and biochemical data showed the stability of gene in advanced generation with enhanced β-carotene levels.

Other associated carotenoids including lutein, zeaxanthin and β-cryptoxanthin have been also found to increased by 10-30 folds in transgenics as compared to control. Several transgenic events of groundnut were found to accumulate high levels of β-cryptoxanthin (0.51-9.45 μg/g). Semi-quantitative RT-PCR was performed at different seed stages of control and selected transgenic events indicated that the products of lycopene cyclase and phytone synthase genes were found to be present in mature seeds of transgenic plants and absent in the control, whereas phytoene desaturase was present in both

types, although the level of expression varied at different stages of seed development. The second generation transgenic events carrying the *Zm psy1* and tomato β-*lycopene cyclase gene* have also been developed where, β-carotene levels were enhanced multi folds (0.75- 5.5 μg/g) when compared to the untransformed controls (0.01-0.03 μg/g) (Sharma KK unpublished results).

Pigeonpea

At ICRISAT, initially a single *psy1* gene from maize was used to develop transgenic pigeonpea for enhanced level of β-carotene using the *Zm psy1* gene driven by the oleosin promoter through *Agrobacterium*-mediated genetic transformation. Over 140 putative transgenic pigeonpea events with maize *psy1* were developed and characterized at the molecular level for the integration and expression of the transgenes (Sharma KK unpublished results). Total carotenoids content in seeds from the primary T_0 putative transgenic pigeonpea plants were estimated spectrophotometrically and two-fold increases in total carotenoid content were observed in several transgenic events over the non-transgenic (control) pigeonpea plant. These 11 events showed 2 to 3-folds increases in β-carotene levels (6-11 μg/g in transgenic events, in contrast to 2 μg/g in the untransformed control) evidenced using HPLC analysis. Studies also indicated that the transgenics pigeonpea events had much higher lutein content over the controls amongst the individual carotenoids. Besides β-*lycopene cyclase* gene was cloned from tomato and used in combination with the *psy1* gene under the control of CaMV35S promoter to further improve β-carotene levels in transgenic groundnut. Efforts are underway to develop marker-free pigeonpea transgenic plants carrying both maize *psy1* and tomato β-*lyc* genes to meet the target levels of β-carotene in this important pulse crop (Sharma KK unpublished results).

Vitamin C

Transgenic *Arabidopsis* over-expressing D-galacturonic acid reductase (*GalUR)* driven by constitutive 35S CaMV promoter has demonstrated its ability to increase vitamin C levels by at least 2-3 folds over non-transgenic control (Agius et al 2003), This is also possible in other plant species where D-galacturonic acid is present in all plant species as cell wall component of pectins. Chen et al (2003), observed that overexpression of DHAR (dehydroascorbate reductase) in plants increase the level of ascorbic acid through improved ascorbate recycling, a DHAR cDNA from wheat was isolated and expressed in tobacco and maize, where DHAR expression was increased up to 32- and 100-folds, respectively. The increase in DHAR expression increased foliar and kernel ascorbic acid levels 2- to 4-folds and significantly increased the ascorbate redox state in both tobacco and maize. In addition, the level of glutathione, the reductant used by DHAR, also increased. These results demonstrate that increasing the expression of the enzyme responsible for recycling ascorbate can elevate the content of vitamin C in plants.

Vitamin E

Seed-specific expression of genes encoding tocochromanol pathway in soybean increased the total tocochromanols up to 15-folds in the seeds of best performing events. Although control soybean seeds contain only traces of tocotrienols, these transgenic soybeans accumulated up to 94% of their tocochromanols as tocotrienols (Cahoon et al 2003; Van Eenennaam et al 2003). The overexpression of γ-tocopherol methyltransferase has changed the composition of α-tocopherol in *Arabidopsis* seeds (Shintani and Penna 1998).

BIOAVAILABILITY AND COST-EFFECTIVENESS OF BIOFORTIFIED CROPS

The degree to which the amount of an ingested nutrient is absorbed and available to the body is called bioavailability. The bioavailability of micronutrients in plant foods can be greatly affected by the composition of the diet consumed. Various food processing techniques, meal components and meal preparation techniques can modify plant foods in ways that either promote or reduce the amount of bioavailable micronutrients in these foods. The type of food matrix in which carotenoids are located determines their bioavailability to a great extent. Fat-soluble

compounds reduce carotenoid absorption and interaction among carotenoids may also result in a reduced carotenoid bioavailability. The principal cost components for biofortification relate to the research needed to develop biofortified varieties and their implementation. Because an agricultural research system is needed to develop modern varieties of staple foodstuffs, the research costs are essentially the incremental costs of enhancing micronutrient density. These research costs are likely to be the single largest cost component of biofortification and are a one-time investment, incurred at the outset. It is estimated that costs associated with plant breeding will average about $400,000 per year per crop over a 10-year period, globally (Nestel et al 2006). Biofortification is a worthwhile investment even where the calculated benefits do not include the enhanced incomes that may result after adopting agronomically superior biofortified varieties and is likely to be more cost effective than supplementation and other fortification programs.

CONCLUSION

Biofortification provides a feasible means of reaching malnourished populations in relatively remote rural areas where markets fail to reach, given that they are largely sustained by subsistence agriculture. Food fortification makes sense as part an integrated food systems approach for reducing malnutrition. It addresses the root causes of micronutrient malnutrition, targets the poorest people, and uses built-in delivery mechanisms that are scientifically feasible and cost-effective, and complement other ongoing methods of dealing with micronutrient deficiencies. It is an obvious first step in enabling rural households to improve family health and nutrition in sustainable ways. A relative lack of a concentrated food processing chain, less developed commercial markets, and relatively low consumer awareness and demand have hindered the same application of the intervention in the transitional, and even more, in the least developed countries until quite recently. The nutritional fortification of cereals is still in its infancy. Little is known about the physiological and biochemical mechanisms that control micronutrient accumulation in plants. Further research is needed to understand the mechanisms of uptake and transport to redirect nutrients for efficient accumulation in cereal seeds to be successful, biofortification strategies must combine screening of germplasm for enhanced micronutrient content with breeding and genetic engineering strategies to improve the nutritional quality of cereals. Much basic research in this area is still required before future applications can be successful. Because of its social impact, and public concerns about the genetic engineering of food crops, biofortification has also become an important topic in the socio-economic literature. It is worth exploring as it has an immense potential in developing nutritious crops, which will serve as a promising tool for improved human health.

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