Nutrient Biofortification of Food Crops

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Abstract

Plant-based foods offer an array of nutrients that are essential for human nutrition and promote good health. However, the major staple crops of the world are often deficient in some of these nutrients. Traditional agricultural approaches can marginally enhance the nutritional value of some foods, but the advances in molecular biology are rapidly being exploited to engineer crops with enhanced key nutrients. Nutritional targets include elevated mineral content, improved fatty acid composition, increased amino acid levels, and heightened antioxidant levels. Unfortunately, in many cases the benefits of these "biofortified" crops to human nutrition have not been demonstrated.

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INTRODUCTION

The relationship between diet and health is an active area of research; however, a substantial body of evidence already indicates that food components can influence physiological processes (102). Thus, functional foods (**Table 1**) are of increasing interest in the prevention and/or treatment of diseases (105). However, for much of the world's population, plant-based foods are used to simply provide essential nutrients (7). Some nutrients are required in large amounts, but others, such as iron (Fe), zinc (Zn), copper (Cu), iodine (I), and selenium (Se), are required in trace amounts because higher levels could be harmful (63). Because each plant has a different nutritional profile, micronutrient malnutrition (MNM), the dietary insufficiency of one or more micronutrients, remains a concern in populations that do not have a balanced diet. MNM has been significantly alleviated in some countries as a result of programs that fortify processed foods. Nevertheless, fortification efforts have not been as successful in countries with limited industrial agriculture, food processing and distribution networks (114). The global significance of MHM—also known as hidden hunger—has been a focus of the nutrition community and has also become a mandate among development agencies and governments.

Biofortification is the process by which the necessary daily micronutrients are delivered directly to staple crops (11, 12). Plants are versatile biochemical factories, capable of synthesizing a nearly full complement of essential dietary micronutrients (the exceptions being vitamins D and B_{12}; 7). Regrettably, the plant-based foods (rice, wheat, cassava and maize) most abundantly consumed by at-risk populations contain levels of several micronutrients that are insufficient to meet minimum daily requirements. Furthermore, often these nutrients are unevenly distributed among plant parts (155). For example, iron content is high in rice leaves but low in the polished rice grain. Similarly, provitamin A carotenoids are only present in rice leaves. Biofortification efforts are directed toward increasing the levels of specific, limiting micronutrients in edible tissues of crops by combining crop management, breeding, and genetic approaches.

Extensive programs are underway to enrich the nutrient content of plants both to improve health and to prevent disease (**Table 2**). Recent reviews abound and have highlighted this technology and the various risk factors this entails (49, 64, 80, 144, 146). This review examines the current strategies for the biofortification of crops, including mineral fertilization and conventional breeding, but focuses predominately on the recent advances in biotechnology to alter plants to improve nutritional content.

Table 1 Examples of plant components with suggested functionality. The examples shown do not constitute an all-inclusive list (75, 105)

Class/component	Examples of sources	Potential health benefit
Carotenoids		
α- and β-Carotenes	Carrots, pumpkins, sweet potatoes, kale	Neutralizes free radicals that may cause damage to cells
Lutein	Kale, collard greens	Contributes to maintenance of healthy vision
Lycopene	Tomato, watermelon	May reduce risk of prostate cancer
Zeaxanthin	Citrus, maize	Contributes to maintenance of healthy vision
Dietary fiber		
Insoluble fiber	Whole-grain barley, vegetable peels	May reduce risk of breast and/or colon cancer
Soluble fiber[a]	Fruits, vegetables, legumes	May reduce risk of cardiovascular disease (CVD)
Whole grains[a]	Cereal grains	May reduce risk of CVD
Fatty acids		
ω-3 fatty acids (DHA/EPA)	Flaxseed oil, walnuts	May reduce risk of CVD and improve mental and visual functions
ω-6 fatty acids	Safflower oil, corn oil, soybean oil	May reduce risk of CVD and improve mental and visual functions
Flavonoids		
Anthocyanidins	Blueberries, red onions	Neutralizes free radicals, may reduce risk of cancer
Flavanols: catechins, tannins	Tea (green, black, tannins), red apple	Neutralizes free radicals, may reduce risk of cancer
Flavanones	Lemon, grapefruit	Neutralizes free radicals, may reduce risk of cancer
Flavones	Parsley, thyme	Neutralizes free radicals, may reduce risk of cancer
Glucosinolates, indoles, isothiocyanates		
Sulforaphane	Mustard, horseradish	Neutralizes free radicals, may reduce risk of cancer
Phenolics		
Stilbenes (resveratrol)	Red grapes, pecans	May reduce risk of degenerative diseases, heart disease, and cancer; may have longevity effect
Caffeic acid, ferulic acid	Fruits, vegetables, citrus	Antioxidant-like activities; may reduce risk of degenerative diseases, heart disease, and eye disease
Plant stanols/sterols		
Stanol/sterol ester[a]	Maize, soy, wheat	May reduce risk of coronary heart disease by lowering blood cholesterol levels
Phytoestrogens		
Isoflavones (daidzein, genistein)	Soybean, okra	May reduce menopause symptoms, such as hot flashes; may reduce osteoporosis and CVD
Lignans	Flax seeds, kale, apricots	May protect against heart disease and some cancers, may lower LDL cholesterol, total cholesterol, and triglycerides
Sulfides/thiols		
Diallyl sulfide	Onion, garlic, olive	May lower LDL cholesterol, helps to maintain healthy immune system
Tannins		
Proanthocyanidins	Cranberry, cocoa	May improve urinary tract health, may reduce risk of CVD and high blood pressure

[a] U.S. Food and Drug Administration-approved health claim established for component.

Table 2 Examples of crops with nutritionally improved traits intended to provide health benefits for consumers and animals. This list excludes protein/starch functionality, shelf life, taste/aesthetics, fiber quality, and allergen and toxin-reduction traits (75, 105)

Trait	Crop (trait detail)	References
Protein and amino acids		
Protein quality and level		
	Canola (amino acid composition)	(124)
	Maize (amino acid composition; protein↑)	(109, 148, 150)
	Potato (amino acid composition; protein↑)	(7, 89)
	Rice (protein↑; amino acid composition)	(81)
	Soybean (amino acid balance)	(38, 121)
	Sweet potato (protein↑)	(42)
Essential amino acids		
	Maize (Lys↑, Met↑)	(3, 88)
	Potato (Met↑)	(153)
	Sorghum (Lys↑)	(154)
	Soybean (Lys↑, Trp↑)	(44, 53)
Oils and fatty acids		
	Canola (lauric acid↑; + ω-3 fatty acids; 8:0 and 10:0 fatty acids↑; lauric and myristic acids↑; oleic acid↑)	(3, 30, 34, 50, 51, 78, 124)
	Cotton (oleic acid↑, oleic + stearic acids↑)	(21, 91, 108)
	Grass, legumes (↓trans-fatty acids)	
	Linseed (+ ω-3 and ω-6 fatty acids)	(1)
	Maize (oil↑)	(150)
	Oil palm (oleic acid↑ or stearic acids↑, oleic acid↑, +palmitic acid↓)	(77, 113)
	Rice (α-linolenic acid↑)	(6)
	Soybean (oleic acid↑)	(84, 122)
Carbohydrates		
Fructans		
	Maize (fructan↑)	(19)
	Potato (fructan↑)	(71)
Inulin		
	Potato (inulin↑)	(70)
Starch		
	Rich (amylase↑)	(23, 128)
Micronutrients and functional metabolites		
Vitamins and carotenoids		
	Canola (vitamin E↑)	(131)
	Maize (vitamin E↑, vitamin C↑)	(18, 22, 123)
	Mustard (+β-carotene)	(129)
	Potato (β-carotene and lutein↑)	(41)
	Rice (+β-carotene)	(149)
	Strawberry (vitamin C↑)	(4)
	Tomato (folate↑, phytoene and β-carotene↑, lycopene↑, provitamin A↑)	(32, 37, 43, 47, 96, 125)

(*Continued*)

Table 2 (*Continued*)

Trait	Crop (trait detail)	References
Functional secondary metabolites		
	Apple (+stilbenes)	(134)
	Alfalfa (+resveratrol)	(73)
	Maize (flavonoids↑)	(152)
	Potato (anthocyanin and alkaloid glycoside↓, solanin↓)	(94)
	Rice (flavonoids↑, +resveratrol)	(130, 132)
	Soybean (flavonoids↑)	(152)
	Tomato (+resveratrol, chlorogenic acid↑, flavonoids↑, stilbene↑); (anthocyanins↑)	(17, 57, 100, 106, 125)
	Wheat (caffeic and ferulic acids↑, +resveratrol)	(137)
Mineral availabilities		
	Alfalfa (phytase↑)	(8)
	Carrot (calcium↑)	(98, 112)
	Lettuce (iron↑)	(58)
	Rice (iron↑)	(93)
	Maize (phytase↑, ferritin↑)	(40)
	Soybean (phytase↑)	(35)
	Wheat (phytase↑)	(15)

BIOFORTIFICATION THROUGH FERTILIZER APPLICATION

Although simple and inexpensive, the application of fertilizers containing essential mineral micronutrients is complicated by several factors, such as the application method, soil composition, mineral mobility in the plant, and its accumulation site (155). Therefore, this strategy has been successful in only limited cases and in particular geographical locations.

Iodine and selenium are mobile in soil and in plants, thus biofortification with iodine and selenium fertilizers has been particularly successful (28, 66). Because Zn is also mobile in the soil, applications of $ZnSO_4$ can also increase yield and Zn concentrations in cereals and legumes (146).

In contrast, Fe has a low mobility in soil because $FeSO_4$ is rapidly bound by soil particles and converted into Fe(III); therefore, Fe fertilizers have not been successful in biofortification efforts (63). Furthermore, large quantities of metals applied to soils can be deleterious to plant growth and other soil organisms (62).

Micronutrient fertilizers often must be applied regularly and are costly as well as potentially damaging to the environment. Overall, such strategies are applicable to specific crops and mineral scenarios but cannot be universally applied as a strategy to boost the nutritional quality of foods.

BIOFORTIFICATION THROUGH CONVENTIONAL BREEDING

Plants often show genetic variation in essential nutrient content, which then allows breeding programs to be used to improve the levels of minerals and vitamins in crops (20, 54, 97, 145). For example, different rice genotypes show a 4-fold variation in iron and zinc levels, and up to a 6.6-fold variation has been reported in beans and peas (60, 62). Given that this approach uses intrinsic properties of a crop, there are few regulatory constraints. Furthermore, this approach has the blessing of vocal opponents to genetic engineering. Because this approach is likely to be the most expedient method to improve plants, several international organizations have initiated programs to improve the nutritional content of crops through breeding programs. For example, Harvest-Plus is investing $14 million annually to boost three key nutrients—vitamin A, iron, and zinc—in 12 target crops, relying almost exclusively on conventional breeding (114).

Conventional breeding has several significant disadvantages when compared to transgenic approaches (12). Breeding strategies rely on the sometimes limited genetic variation present in the gene pool. In some cases, this can be overcome by crossing to distant relatives and thus moving the trait slowly into the commercial cultivars. Such a strategy could be used to improve selenium levels in wheat, because today's bread wheats show little variation in selenium content whereas wild wheat has higher levels (95). Alternatively, new traits can be introduced directly into commercial varieties by mutagenesis (119). Mutagenesis has been used to generate lysine-rich maize lines, and mutations to remove substances that impede bioavailability have improved several cereals and legumes (105; **Table 2**).

TRANSGENIC APPROACHES FOR BIOFORTIFICATION

In the absence of genetic variation in nutrient content among varieties, breeders have nothing to work with. This is where transgenic approaches can be a valid alternative (14, 155). Nutritional genomics studies the relationship between genomes, nutrition, and health (13, 31). The ability to rapidly identify and characterize gene function and then utilize these genes to engineer plant metabolism has been a driving force in recent biofortification efforts (24). This was made possible by the rapid development of whole-genome sequencing, high-throughput physical mapping, global gene expression analysis, and metabolite profiling in a variety of organisms (53). Furthermore, as is noted throughout this review, pathways from bacteria and other organisms can also be introduced into crops to exploit alternative pathways for metabolic engineering (105). Thus, these technologies provide a powerful tool that is unconstrained by the gene pool of the host (29). In addition, the genetic modifications can be targeted to the edible portions of commercial crops (5, 129, 148). As shown in **Table 2**, numerous crops have been genetically modified with macronutrient and micronutrient traits that may provide benefits to consumers and domestic animals (105).

Although the possibilities associated with transgenic approaches keep plant biologists optimistic, regulatory hurdles associated with this technology make commercial applications difficult (49, 75, 80, 117, 144). Nearly all transgenic plants have patented or patentable inventions associated with them; however, there has been a movement to work around patents to deliver biotechnology to the poor farmers of the world (117). Regrettably, the current political and economic landscape is not receptive to this technology being widely applied to a host of different crops. Even with these current limitations, the potential for genetic modifications to alleviate hunger warrants advocacy of this technology among both scientists and citizens.

ANTIOXIDANTS

The consumption of fruits and vegetables has been shown to increase plasma antioxidant levels in human subjects (**Table 1**). Fruits and vegetables contain a wide range of antioxidants including anthocyanins and carotenoids such as lycopene and β-carotene and vitamins C and E (10, 151). Carotenoids, along with a variety of other compounds including sterols and tocopherols, are derived from the general isoprenoid biosynthetic pathway. Colored carotenoids are found in fruits, flowers, and roots, where they probably act as attractants to pollinators and for seed dispersal.

ANTHOCYANINS

Anthocyanins are water-soluble pigments that may appear red, purple, or blue. They belong to a parent class of molecules called flavonoids. Fruits of most tomatoes contain negligible levels of anthocyanins, and previous efforts to boost their levels have caused only modest increases, mostly in the fruit peel, which is often removed during processing. Boosting the levels of two transcription factors that regulate the expression of the genes involved in the entire metabolic pathway increased the anthocyanin content of both the peel and flesh (17). The resulting purple fruit color is maintained after backcrossing into a number of commercial tomato varieties and is not associated with adverse growth or yield (**Figure 1**). In pilot feeding studies, diets containing 10% by weight of the transgenic tomato powder extend the life span of tumorgenic mice. Currently, blackberries and raspberries are among the best sources of dietary anthocyanins, but both are expensive and are consumed in smaller quantities than tomatoes. Potentially, these engineered tomatoes could contribute substantially to the antioxidant levels of human diets.

LYCOPENE

Because tomatoes are the predominate dietary source of lycopene, its formation in the tomato has been the subject of considerable attention (125). Natural mutants of tomato are available, such as a high-pigment variety that has been used in breeding strategies to alter lycopene levels (126). Expression of bacterial genes and yeast genes in transgenic tomatoes has also significantly altered lycopene levels (48, 96).

It is a matter of some debate whether raising the levels of lycopene in fruit will have a major influence on bioavailability (135). Lycopene is fat soluble and as such is poorly absorbed by the body from fresh fruit. The configuration of the lycopene, whether in the cis or trans form, may also alter absorption rates. Furthermore, it remains unclear whether enhanced lycopene in foods can offer benefits to human health that are substantially different from synthetic lycopene.

VITAMIN E

Tocochromanols are a group of four tocopherols and four tocotrienols that collectively constitute vitamin E (18, 33). Although they have similar antioxidant activities in vitro, the individual tocochromanols vary widely in their in vivo vitamin E activities. Through the use of nutritional genomics, a relatively small genetic change can produce a large increase in the vitamin E content of plants (32, 131). Furthermore, coexpressing several of the tocochromanol biosynthetic genes can alter the content and types of tocochromanols. Plant biologists can convert the lower-activity precursors to the highest-activity vitamin E compounds. With this technology, the vitamin E content of seed oil has been increased nearly tenfold. Progress has been made to move the technology to agricultural crops such as soybean, maize, and canola (33).

An additional benefit to this work is that it may lead to foods with a longer shelf life (96). It is thought that foods with higher levels of vitamin E are protected from oxidative stress and that increasing the levels of these compounds may lead to enhanced agricultural productivity as well as improved storage properties for seeds and vegetable oils.

FOLATE

Folate is a generic term for tetrahydrofolate (THF) and its derivates. Folates are B vitamins; the recommended dietary allowance for folate ranges from 400 to 600 μg per day for pregnant women (32). Plant-based foods are the primary source of folate; however, plants vary in their folate levels, and cereals—particularly rice and wheat—contain extremely low folate levels. Folate is a complex molecule that is assembled from three different components: pteridine, para-aminobenzoic acid (PABA), and glutamate (**Figure 2**). These components are synthesized in different compartments within the plant cell, and the folate is synthesized from these precursors within the mitochondria (32).

The genes and enzymes of folate synthesis have been well characterized, enabling metabolic engineering of the pathway, and results from pilot engineering studies in plants (and bacteria) are encouraging. Initially, the first committed enzyme in the ptereidine pathway was highly expressed in transgenic plants, and this resulted in a 100- to 1000-fold increase in pteridine levels but only a 2- to 3-fold increase in folates (37). When exogenous PABA was added to these plants, folate levels could be increased, which suggests that PABA synthesis may limit folate production. In order to increase PABA levels, plants were engineered to express high levels of an enzyme that makes PABA within a separate compartment of the plant cell. These lines contained 20-fold-higher levels of PABA, but the folate levels remained unchanged relative to controls. Interestingly, when PABA-overexpressing plants were crossed to the ptereidine-enhanced lines, the folate levels increased up to 25-fold compared to controls (36). This "stacking" of these two modifications in tomatoes produced transgenic fruit containing 840 μg per 100 grams of tomato fruit, an amount that would be more than sufficient to provide the entire dietary allowance of folate.

Enhanced levels of folate were obtained in transgenic rice through the use of a similar approach of stacking genes for two enzymes in the folate biosynthetic pathway (133). These biofortified rice lines had 100-fold more folate than did controls, and cooking experiments suggest that 100 g of this modified rice may supply the daily dietary allowance of folate.

These folate-biofortification successes in both a monocot and dicot portend the stacking approach being successfully applied to various other crops. Optimization of the metabolic fluxes within these genetically modified (GM) plants and their effects on bioavailability require further investigation.

VITAMIN A

Vitamin A deficiency (VAD) is prevalent among poor persons whose diets are based mainly on rice or other carbohydrate-rich, micronutrient-poor calorie sources (116). Dependence on rice as a predominant food source leads to VAD. According to the World Health Organization, dietary VAD directly affects some 250,000 to 500,000 children every year.

Rice containing provitamin A could substantially reduce VAD. Thousands of rice varieties have been analyzed and none have provitamin A in their rice seeds; thus, rice containing provitamin A can be obtained only through genetic engineering. Rice plants produce β-carotene (provitamin A) in green tissues but not in the edible seed. Even though all required genes to produce provitamin A are present in rice grain, some genes are turned off during development. In transgenic rice lines, genes have been inserted that allow provitamin A to be made in seeds (149). Initially, golden rice required the successful introduction of three genes to encode the additional enzymes, and it is still considered a technical tour de force. This intervention leads to the production and accumulation of β-carotene in the grains. These transgenic grains have a yellow color; the term "golden rice" was coined by a Thai businessman (115). The development of golden rice was featured in newspapers, magazines, documentaries and television shows worldwide. Naturally, critics of biotech foods immediately pointed out the obvious, that, the amount of β-carotene in the initial rice lines was insufficient to have a significant impact on human nutrition (104). Since a prototype golden rice was developed in 1999, new lines with higher β-carotene content have been generated (110). The intensity of the golden color is an indicator of the concentration of β-carotene in the seeds (**Figure 3**). It is conceivable that these new rice varieties will be capable of providing the recommended daily allowance of vitamin A—in the form of β-carotene—in 100 to 200 g of rice, which corresponds to the daily rice consumption of children in rice-based societies.

Recently, this approach has also been successfully applied to "golden potatoes" to obtain carotenoid levels of 114 micrograms per gram dry weight and β–carotene levels of 47 micrograms per gram dry weight (39, 41).

Any "golden food" nutritional studies will not be straightforward (85, 104). The absorption of provitamin A depends on the overall nutritional status, which in turn depends on the diversity of the food consumed. People who eat predominately plain rice or potatoes do not have the luxury of a diversified diet (74). Thus the nutritional impact of golden foods needs to be assessed using diets that will not favor optimal absorption of the nutrient.

The development of golden rice as a commodity is an important gauge for the future of all GM crops. Golden rice is the first GM crop designed to help poor consumers in developing countries, and not farmers and pesticide companies (115). Vocal opponents of this technology believe golden rice is unsafe to eat and that a massive use of this variety would reduce diversity and threaten food security. The current consumer reluctance and rigorous regulatory hurdles have thwarted some commercialization efforts (117). Plant biologists believe that if this technology is to live up to its promise, it is imperative that the quagmire of the current regulatory systems be overhauled so that GM technology can benefit the poor.

OTHER CAROTENOIDS

The ability to regulate ripening in various fruits has direct implications to their carotenoid concentrations. Ripening also alters the development of aroma, flavor, color, and texture components (9). These attributes are influenced by the endogenously produced hormone ethylene in fruits. Even in crops where endogenous ethylene seems to play little if any role as an endogenous regulator, exogenous ethylene will often promote ripening characteristics and can be the target of postharvest strategies designed to accelerate, synchronize, or delay ripening.

Many carotenoid biosynthesis genes have been elucidated through the study of tomatoes (43, 46, 47). Recent insights have pointed to light signal transduction genes regulating carotenoid accumulation in fruits (55, 92). The genes, which regulate sensitivity to normal light regimes, presumably mediate elevated pigment levels to protect against the perceived threat of photo-oxidation. Transgenic tomato experiments confirm that light signal transduction components regulate the accumulation of carotenoids and other antioxidants.

The engineering of ethylene levels in diverse plant species is an active area of research and the regulation of light signaling components in tomatoes will also soon be transferred to other agriculturally important crops (56). However, the signal transduction systems that might fine-tune ripening phenomena according to environmental conditions are still poorly understood. Initial inroads into dissecting regulatory pathways that affect a subset of fruit characteristics, including those that contribute to metabolic content and associated fruit quality, are beginning to emerge.

As mentioned in the discussion of lycopene and vitamin A studies, carotenoids—like many of the other phytonutrients discussed in this review—seem to be health promoting when taken at the physiologic levels found in foods. However, future work is needed to unravel aspects of the absorption, metabolism, and biological functions of these nutrients.

ESSENTIAL AMINO ACIDS

Most crops are deficient in one or more essential amino acids that cannot be synthesized de novo by humans; for example, legumes tend to lack methionine and cysteine, whereas grains tend to be deficient in lysine and threonine (67, 105, 109, 127). Because the majority of the world's population relies on legumes and cereals for their diet, plant biologists have used various methods to increase essential amino acids in these plants (121). For example, expression of storage proteins that contain high levels of desirable amino acids has raised lysine content in rice and wheat (24). Similar approaches have raised essential amino acid content in potatoes (42). However, attempts to raise sulfur-containing amino acids have not been as productive (38). To address these issues, synthetic proteins have been expressed in cassava to match the amino acid requirements for humans.

The inability to predictably alter the amino acid content of target crops reflects the limited availability of free amino acid pools within the edible portion of plants. In higher plants, the synthesis of lysine, threonine, and methionine are under complex feedback control (72). Work has now focused on developing feedback-insensitive enzymes that are controlling these pathways (105). The use of this type of technology has significantly increased the free lysine levels in maize (from 2% to almost 30%). Expression of these altered enzymes has also improved lysine content in canola and soybean and produced a significant increase in tryptophan levels in grains. Obviously, a fundamental concern with each of these manipulations is to ensure that the total amount and composition of storage proteins is not altered to the detriment of the development of the crop.

ESSENTIAL AND VERY-LONG-CHAIN FATTY ACIDS

Genetic modifications of oilseed crops can provide an abundant, relatively inexpensive source of dietary fatty acids (1, 6, 84, 91, 122, 140). Production of these lipids in vegetables could provide an easy mechanism to delivery of healthier products without major dietary modifications (27, 105). Plants have the potential to be valuable sources of the essential fatty acids linoleic acid and linolenic acid as well as very-long-chain polyunsaturated fatty acids (VLC-PUFAs), arachidonic acid (ARA), eicosapenoic acid (EPA), and docosahexaenoic acid (DHA), which are usually found in fish oils.

Given that many of the enzymes involved in fatty acid biosynthesis and degradation have been characterized, there is an abundance of transgenic approaches to the modification of oil and fat content in plants (16, 27). Examples of such modified oils include low- and zero-saturated fat soybean and canola oils, canola oil containing medium-chain fatty acids, high-stearic-acid canola oil, high-oleic-acid soybean oil, and canola oil containing the polyunsatured fatty acid linolenic acid.

Oils abundant in monounsaturated fatty acids provide improved oil stability, flavor, and nutrition qualities. Oleic acid (18:1), a monounsaturate, can provide more stability and health-promoting effects than the polyunsaturates (18:2 and 18:3). Soybeans have been manipulated to contain more than 80% oleic acid (23% is normal) and had a significant decrease in polyunsaturated fatty acids (84). High-oleic-acid soybean oil is more resistant to degradation by heat and oxidation requiring little or no postrefining processing. Soybean oil composed of at least 80% oleic acid, and over 20% less saturated fatty acids than current soybean oil may soon enter the marketplace. These new varieties will produce soybean oil that is more stable, has less need for hydrogenation, and that has a concomitant reduction in transfatty acids. Ingenious methods are being initiated to reduce transfats in livestock products by preventing plant-derived *cis*-polyunsaturated fatty acids from being transformed into saturated transfats in the rumen. Researchers are engineering forage crops with polyoleosin genes from sesame, which should result in triglycerides being encapsulated within self-assembling micelles, thus sealing them off from bacterial activity during transit through the rumen (108).

Elevated expression of VLC-PUFAs has also been obtained in plants by reconstructing a bacterial pathway that is not normally present in plants (27, 135). Generating transgenic plants have three different bacterial genes produced EPA and ABA in vegetative tissues at levels of 3.0% and 6.6% of total fatty acids. The elevated accumulation of VLC-PUFAs did not have deleterious effects on plant growth.

DHA biosynthesis has been obtained by expressing zebra fish and algal genes in the seeds of plants. These transgenic seeds total fatty acids containing up to 0.5% DHA. In soybean, this technology has been further refined to obtain up to 3% DHA levels.

In each of these studies, there has been a striking increase in flux through the VLC-PUFA biosynthetic pathway, leading to the accumulation of ARA and EPA. Unfortunately, the levels of DHA remain low, which suggests the need for further work that analyzes the pathway and the feedback mechanisms employed (135).

MINERAL BIOFORTIFICATION

Although metabolic engineering is most suitable for fortifying plants with organic nutrients, a different approach is required for minerals because they are not synthesized by the plant but rather are "mined" from the immediate environment. Two distinct approaches have been used to improve mineral content: (*a*) increase the efficiency of uptake and transport into edible tissues and (*b*) increase the amount of bioavailable mineral accumulation in the plant (25, 52).

Iron and Zinc

Engineering strategies to increase the mineral content of plants have concentrated primarily on iron and zinc, which are most frequently deficient in human diets (26, 93, 111). In fact, iron deficiencies are the most prevalent nutritional problem in the world today, affecting an estimated 2.7 billion people.

Grasses utilize a different mechanism to obtain Fe(II) than do other plants (76). However, all plants must first take Fe(III) that is abundant in the soils and convert it to Fe(II). Specific transporters are then used to absorb the minerals into the roots and transport the metal in complexes such as nicotianamine, which can chelate Fe(II) and mobilize the mineral to other locations within the plant (61, 120). The increased expression of several of these transport and chelating proteins in transgenic plants promotes metal accumulation. There appears to be some cross-talk between iron- and zinc-transport pathways because plants engineered to increase iron content often also have increased zinc accumulation. This could reflect the enhanced synthesis of nicotianamine, which increases the mobilization of both metals. In fact, manipulation of nicotianamine in plants can double both zinc and iron levels in plants.

The second approach to mineral biofortification is to express recombinant proteins that enable minerals to be stored in a more bioavailable form. Expression of ferritin, an iron-storage protein, in seeds causes a three- to fourfold increase in iron levels (58, 59, 139). Although polishing of rice causes a decrease in mineral levels, ferritin-enhanced rice still has increased iron levels in the transgenic polished rice. Rats fed a diet containing the transgenic rice demonstrate that the iron in the rice had bioavailability equal to that found in diets containing $FeSO_4$ at equal concentrations (101).

The removal of antinutrients from plants can also increase bioavailable mineral content. Phytic acid (also known as phytate) is an antinutrient that can chelate minerals and reduce their bioavailability in the gut (35). A combined approach has been developed that involves the expression of iron-storage proteins and phytase (a fungal enzyme that breaks down phytate); this has been achieved in both rice and maize (40). This combined approach for mineral biofortification should provide maximal levels of bioavailable iron.

Calcium

The low calcium content of most widely consumed vegetables currently makes them a minor contributor to calcium intake for most Americans (143). Calcium present in plant foods exists primarily as a complex, in which it is bound to substances such as oxalate, phytate, fiber, lactate, fatty acid, protein, and other anions (90, 147). Phytic acid is often considered as an antinutrient because it forms insoluble complexes with minerals such as zinc, calcium, magnesium, and iron (2, 65, 118). Furthermore, it is not readily digestible by nonruminant livestock or by humans. This can cause significant problems in the management of phosphorus in livestock production and in human nutrition. One approach to studying the nutritional impact of phytic acid in feed and food, and to studying the biology of phytic acid in plants and seeds, is to isolate low-phytic-acid mutants in such plants as maize. Normally, phytic acid is present in large quantities in maize kernels. Human feeding studies compared the absorption of calcium from tortilla meals prepared from low-phytate maize with that from meals prepared from maize with typical phytate content. Mean fractional absorption of calcium from tortillas prepared from the low-phytate maize was significantly greater than that from tortillas prepared from the control maize. This type of approach serves as a paradigm for the integration of plant biology and human nutritional studies.

Previous studies have strongly suggested that calcium oxalate is unavailable for nutritional absorption (45, 68, 69, 141, 142). In general, calcium absorption appears to be inversely proportional to the oxalic acid content in the food. For example, kale is low in oxalate (2.8 mg/g) whereas spinach is high (105 mg/g). These studies lacked precision because the genetic mechanisms underlying the inherent differences in the plants were too numerous to accurately determine the antinutrient(s). In order to clarify these observational studies, comparisons were made in the calcium absorption from isogenic lines of a forage crop, *Medicago*, which differed in a single gene that mediates oxalate crystal content (**Figure 4**; 99, 103). This experimental platform used genetic analysis and mice fed extrinsically and intrinsically labeled diets to show that plants lacking calcium oxalate crystals are better sources of bioavailable calcium.

Plant calcium transporters have been manipulated to increase calcium levels of edible

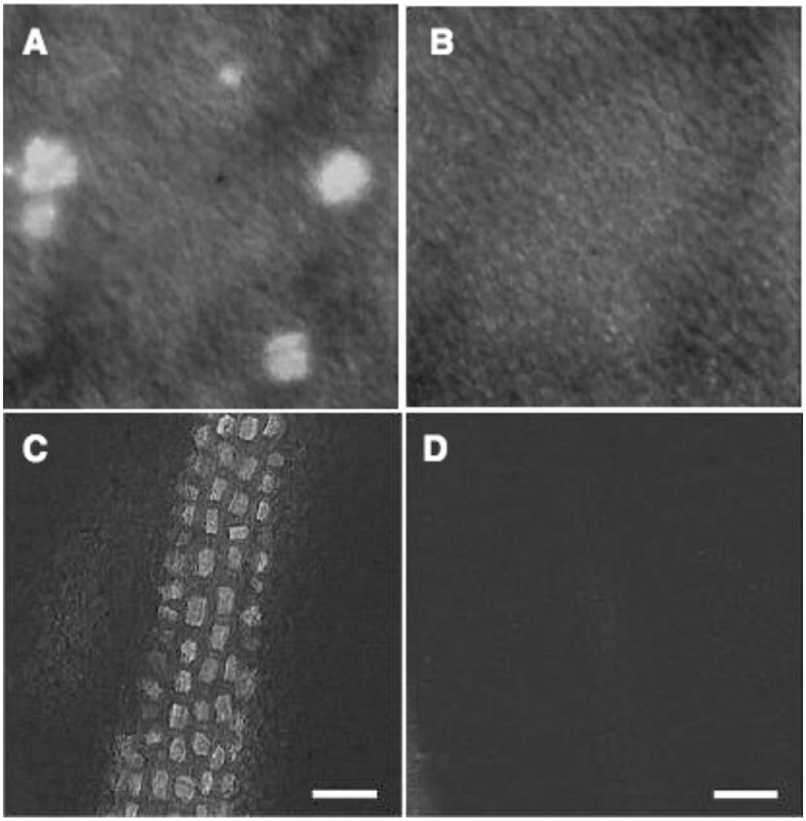

Figure 4

Oxalate crystal development in plants. Oxalate crystal development in (*a*) spinach, (*b*) kale, (*c*) *M. truncatula*, and (*d*) an oxalate mutant of *Medicago*. These plants were six weeks old. Leaves were cleared in acetone and visualized between crossed polarizers using a light microscope. Bar = 30 μm. (Figure used with permission from *Plant Molecular Biology*.)

roots such as carrots (74). Modified carrots expressing high levels of a deregulated transporter accumulate almost twofold more calcium in the edible part compared to control plants, without perturbing growth, development, or fertility, under controlled lab conditions. Feeding trials using these labeled carrots demonstrated that calcium absorption was significantly increased in both mice and humans with diets containing the modified carrots (98).

Interestingly, not all the increased calcium in the transporter-modified carrots was bioavailable. This may be because a fraction of the extra calcium is bound to antinutrients within the carrot. However, the modified carrots are a better source of calcium because total calcium absorbed was higher. This should serve as a cautionary example for scientists who assume that all increases in nutrient content directly equate to increased bioavailability.

IMPACT ON PLANT PRODUCTIVITY AND THE ENVIRONMENT

Assessments of the potential alterations in plant metabolism following biofortification efforts have rarely been analyzed. Altering metabolic fluxes through a specific pathway may affect plant growth and productivity. For example, changes in metal content may alter enzyme activities and metabolism. It is thus imperative to establish whether a specific alteration in plant metabolism is cost effective. Useful biofortification efforts should increase nutrient content while maintaining low cultivation and production costs. Fortunately, improved metabolomic and metabolic modeling techniques should facilitate such analysis (64).

The experimental parameters used to test genetically modified foods should resemble clinical trials with a novel pharmacological agent. Interactions with other nutrients in the plant matrices, potential allergic responses of the consumer, and alterations of plant stress responses are some of the parameters that need to be determined. Interestingly, removal of the antinutrient calcium oxalate crystals causes these modified plants to be more nutritious but reduces the plant's defense to insect chewing (86). The plant is more nutritious for pathogens, too! In these early days of genetically modified foods for health, the scientific community's most important task is to be painstakingly thorough in the safety analyses of these foods before they become readily available to consumers.

MEASURING NUTRITIONAL PARAMETERS

Initially, biofortified foods need to be analyzed using established nutritional phenotypes. Regrettably, a lack of nutritional assessment in biofortified foods is the norm rather than the exception. A three-step process has been proposed to evaluate biofortified foods (83). Tests of nutrient bioavailability initially need to be conducted in the laboratory. Various genotypes of modified foods can be screened using in vitro cell-culture systems or animal models. Second, comprehensive long-term feeding trials need to be conducted to test efficacy among various target populations. The final trial will involve evaluating the nutritional, health, agricultural, and environmental impact of the novel foods on the community. These tests will require a multidisciplinary team that includes consumers, policy makers, health leaders, and plant and nutritional scientists.

While we await standard nutritional evaluations, other promising new assays may soon be able to measure the functionality of genetically modified foods. The ionome is the mineral nutrient (dietary minerals) and trace elements found in any biological material. Measuring the ionome of various tissues could be an important tool when analyzing the effectiveness of various foods (87). Inductively coupled plasma optical emission spectroscopy or inductively coupled plasma mass spectrometry can both be effectively used for ionome measurements of plant or animal tissues. An interesting future direction for these high-throughput measurements will be to monitor how the ionome of various tissues in animals responds to biofortified foods (with altered ionomes). Furthermore, can these changes in the animal ionome be used to predict metabolic changes?

The plethora of emerging mice mutants provides a reservoir of promise for testing nutritional intervention strategies (138). Can a mutant phenotype in an animal be rescued with a diet consisting of a particular biofortified food? As previously mentioned, diets high in anthocyanin tomatoes can extend the life span of cancer-susceptible mice (17). Also, dietary folate supplementation, when properly timed, may suppress the development of tumors (82). Judicious use of animal and plant resources portends the use of biofortified foods, rather than phytochemical extracts, to achieve dietary intervention strategies. These improved foods may be more widely adopted in preventive medicine strategies than in supplements, which are often viewed in the same way as conventional medicines.

Biosensors are analytical tools consisting of a biologically active material used in close conjunction with a device that will convert a biochemical output into a quantifiable signal. In the future, biosensors may be used to measure nutrient content in animal models (107). Could an iron sensor be used to monitor the efficacy of a particular biofortified rice variety similar to ferritin-modified rice? Although this technology is still in a few years off, one can't help but be enthused about the potential use of imaging technology to trace in real time specific nutrients throughout a transgenic animal fed biofortified foods.

NUTRITIONAL SCIENCE MEETS AGBIOTECHNOLOGY

Demand for any biofortified food must be strong enough to drive the product through complicated developmental stages and to offset associated cost increases (117). For this to occur, the health benefits of the foods must be readily apparent to the consumer. As mentioned at the beginning of this review, traditional breeding approaches will find widespread acceptance and can be used to enhance the nutritional qualities of foods (11, 62, 79). Recently, breeding and molecular genetics were elegantly combined to characterize an important wheat gene associated with grain protein, Zn, and Fe content (136). Wild durum (pasta) wheat has shorter grain maturation periods and higher protein, zinc, and iron contents than do domesticated wheats. The version of the gene found in a wild ancestor of durum wheat was isolated, sequenced and compared to the version in modern domesticated wheats. The domesticated wheats have an inactive form of the gene, thus explaining their lower nutrient content relative to the wild durum wheat. The active version can now be incorporated by breeding or genetic engineering to increase the protein, zinc, and iron contents of domesticated wheats. This is likely an example of the optimal experimental approach in biofortification, where research is directed primarily at breeding, and genetic modifications will occur only when necessary.

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DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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Figure 1

Enrichment of tomatoes with anthocyanins. Cross section of ripe wild-type and anthocyanin-enriched tomatoes (photograph from Sue Bunneywell).

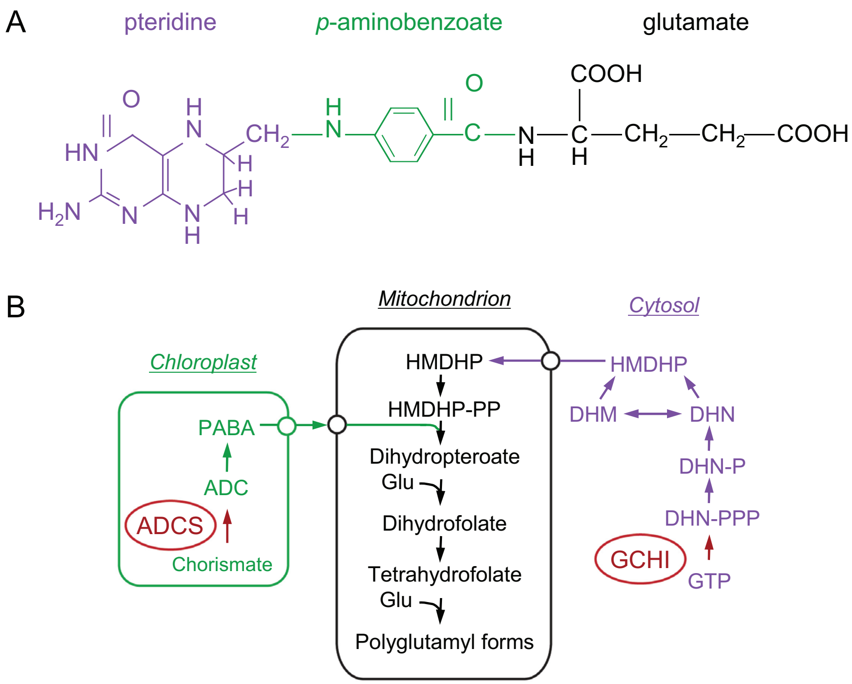

Figure 2

Structure and biosynthesis of folates in plants. (*a*) The chemical structure of monoglutamyl tetrahydrofolate is shown. The pteridine-, para-aminobenzoic acid (PABA)-, and glutamate-derived moieties are color coded. (*b*) The plant folate biosynthetic pathway is shown. The pteridine pathway leading to hydroxymethyldihydropterin (HMDHP) is shown in purple, the pathway leading to p-aminobenzoate is shown in green, and steps localized in the mitochondria are in black. Open circles indicate possible transporters. Red arrows indicate the two enzymes (GCHI and ADCS) engineered to increase folate levels (34, 35) DHN, dihydroneopterin; -P, monophosphate; -PP, pyrophosphate; -PPP, triphosphate; DHM, dihydromonapterin; GTP, guanosine triphosphate. (Figure used with permission from *Proceedings of the National Academy of Sciences USA*.)

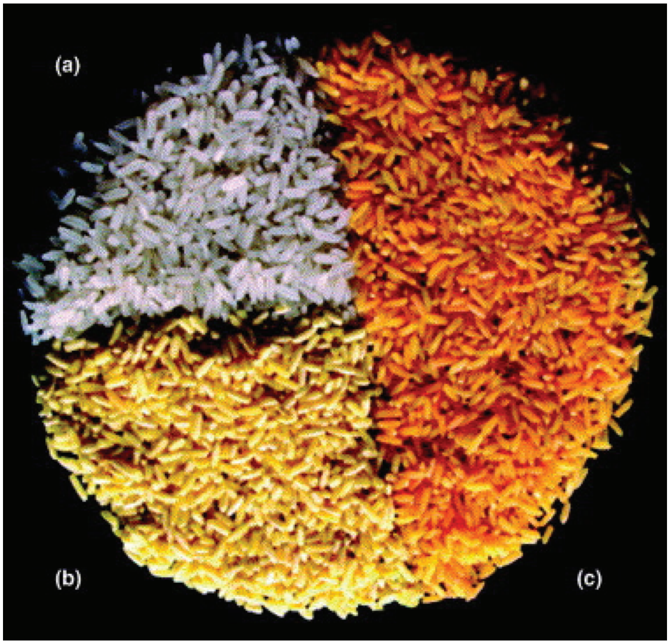

Figure 3

Golden rice colors. (*a*) Wild-type rice; (*b*) the first generation of golden rice; (*c*) the second generation of golden rice. The image clearly shows the progress made since the proof-of-concept stage of golden rice. The new generation contains β-carotene levels that may be able to provide an adequate amount of provitamin A in normal children's diets in Southeast Asia. (Figure used with permission from *Trends in Plant Science*.)

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Errata

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