Plant Biotechnology: The Answer to your Nutrition Needs!

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At its most basic level, food is the source of nutrition to meet daily requirements but is now taking on an ever-greater role in the quest for health optimization. The latter focus is a luxury that is primarily the purview of an affluent society and has little relevance in many areas where mere survival is the driving force. From the basic-nutrition perspective, there is a clear dichotomy in demonstrated need between different regions and socioeconomic groups, the starkest being addressing injudicious consumption in the developed world and under-nourishment in less developed countries (LDCs). Both extremes suffer from forms of malnourishment, one through inadequate supply, the other, in many but not all instances, through inappropriate choices, the latter often influenced by economic considerations. From the food deserts of inner cities to the real barren wastelands of many regions, access to a healthy diet is a challenge. Dramatic increases in the occurrence of obesity, cardiovascular disease, diabetes, cancer and related ailments in developed countries are in sharp contrast to chronic under-nutrition and genuine malnutrition in many LDCs. Both problems require a modified food supply and the tools of biotechnology, while not the sole solution, do have a significant part to play.

Agriculture as a Tool

Worldwide, plant-based products comprise the vast majority of human food intake, irrespective of location or financial status (Mathers, 2006). In some cultures, either by design or default, plant-based nutrition comprises almost 100% of the diet. Given this, one can deduce that significant nutritional improvement can be achieved via modifications of staple crops. New and innovative techniques will be required to improve the efficiency of the global agriculture sector to ensure an ample supply of healthy food. To confound this situation, the inequity between the affluent and developing countries will continue to grow and only a handful of technologies are sufficiently scale-neutral to help with redressing this imbalance. In October 2009, the United Nations' Food and Agriculture Organization (FAO) determined that farming in developing countries needs $83 billion of annual investment for production to feed the world in 2050. The 2008 World Bank Development Report emphasized that, "Agriculture is a vital development tool for achieving the Millennium Development Goals that call for halving by 2015 the share of people suffering from extreme poverty and hunger" (World Bank, 2008). The Report notes that three out of every four people in developing countries live in rural areas and most of them depend directly or indirectly on agriculture for their livelihoods. It recognizes that overcoming abject poverty cannot be achieved in sub-Saharan Africa without a revolution in agricultural productivity for resource-poor farmers, many of whom are women.

The first generation of the products commercialized from one of those technologies, namely biotechnology, were crops focusing largely on input agronomic traits primarily in response to biotic stress. The coming generations of crop plants can be grouped into four broad areas. The present and future focus is on continuing improvement of agronomic traits such as yield and abiotic stress resistance in addition to the biotic stress tolerance of the present generation; crop plants as biomass feedstocks for biofuels and "bio-synthetics"; value-added output traits such as improved nutrition and food functionality; and plants as production factories for therapeutics and industrial products. Developing and commercializing plants with these improved traits involve overcoming a variety of technical, regulatory and perception challenges inherent in perceived and real challenges of complex modifications. Both the panoply of traditional plant-breeding tools and modern biotechnology-based techniques will be required to produce plants with the desired quality traits. Tables 1a–d present examples of crops that have already been genetically modified with macro- and micronutrient traits that may provide nutritional benefits.

The Food/Health Correlation

Although the correlative link between food and health, beyond meeting basic nutrition requirements, has been unequivocally proven only in a number of cases, a growing body of evidence indicates that food components can influence physiological processes at all stages of life. Nutrition intervention from a functionality perspective has a personal dimension. Determining individual response is at least as complex a challenge as the task of increasing or decreasing the amount of a specific protein, fatty acid, or other com-

TABLE 1A. EXAMPLES OF CROPS IN RESEARCH AND/OR DEVELOPMENT WITH NUTRITIONALLY IMPROVED TRAITS INTENDED TO PROVIDE HEALTH BENEFITS FOR CONSUMERS AND ANIMALS[1].

Trait	**Crop (trait detail)**	**Reference**
Protein and amino acids		
Protein quality and level	Bahiagrass (protein↑)	Luciani *et al.*, 2005
	Canola (amino acid composition)	Roesler *et al.*, 1997;
	Maize (amino acid composition; protein↑)	Cromwell *et al.*, 1967; O'Quinn *et al.*, 2000; Yang *et al.*, 2002; Young *et al.*, 2004
	Potato (amino acid composition; protein↑)	Yu and Ao 1997; Chakraborty *et al.*, 2000; Li et al., 2001; Atanassov *et al.*, 2004
	Rice (protein↑; amino acid)	Katsube *et al.*, 1999
	Soybean (amino acid balance)	Dinkins *et al.*, 2001; Rapp, 2002
	Sweet potato (protein↑)	Prakash *et al.*, 2000
	Wheat (protein↑)	Uauy *et al.*, 2006
Essential amino acids	Canola (lysine↑)	Falco *et al.*, 1995
	Lupin (methionine↑)	White *et al.* 2001
	Maize (lysine↑; methionine↑)	Lai and Messing, 2002;
	Potato (methionine↑)	Zeh *et al.*, 2001
	Sorghum (lysine↑)	Zhao *et al.*, 2003
	Soybean (lysine↑; tryptophan↑)	Falco *et al.*, 1995;
		Galili *et al.*, 2002

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[1] Excludes protein/starch functionality, shelf life, taste/esthetics, fiber quality and allergen/toxin-reduction traits; modified from ILSI (2004a, b; 2008).

Table 1b. More examples of crops in research and/or development with nutritionally improved traits intended to provide health benefits for consumers and animals.

Trait	Crop (trait detail)	Reference
Oils and fatty acids		
	Canola (lauric acid↑; γ-linolenic acid↑; + ω-3 fatty acids; 8:0 and 10:0 fatty acids↑; lauric + myristic acid↑; oleic acid↑)	Dehesh et al., 1996; Roesler *et al.*, 1997; Del Vecchio, 1996; Froman and Ursin, 2002; James *et al.*, 2003; Ursin, 2003
	Cotton (oleic acid↑; oleic acid + stearic acid↑)	Chapman *et al.*, 2001; Liu *et al.*, 2002
	Linseed (+ ω-3 and -6 fatty acids)	Abbadi *et al.*, 2004
	Maize (oil↑)	Young *et al.*, 2004
	Oil palm (oleic acid↑ or stearic acid↑; oleic acid↑ + palmitic acid↓)	Jalani *et al.*, 1997; Parveez, 2003
	Rice (α-linolenic acid↑)	Anai *et al.*, 2003
	Soybean (oleic acid↑; γ-linolenic acid↑)	Reddy and Thomas 1996; Kinney and Knowlton 1998;
	Safflower (γ-linolenic acid GLA↑)	BNET, 2008

ponent of the plant itself (Brigelius-Flohe and Joost, 2006). There is also evidence that early food regimes can affect later life health; for example, some children who survived famine conditions in certain regions of Africa grew into adults battling obesity and related problems presumably due to the selective advantage of the thrifty gene in their early food-stressed environment becoming a hazard during more abundant times especially if later diets are calorie dense.

Functional-food components are of increasing interest in the prevention and/or treatment of a number of the leading causes of death including, but not limited to, cancer, diabetes, cardiovascular disease, and hypertension. Many food components are known to influence the expression of both structural genes and transcription factors in humans (Go *et al.*, 2005; Mazzatti *et al.*, 2008). Examples of these phytochemicals are listed in Tables 2a, b; the large diversity suggests that the potential impact of phytochemicals and functional foods on human and animal health is worth examining as targets of biotechnology efforts.

TABLE 1C. MORE EXAMPLES OF CROPS IN RESEARCH AND/OR DEVELOPMENT WITH NUTRITIONALLY IMPROVED TRAITS INTENDED TO PROVIDE HEALTH BENEFITS FOR CONSUMERS AND ANIMALS.

Trait	Crop (trait detail)	Reference
Carbohydrates		
Fructans	Chicory, (fructan↑; fructan modification)	Smeekens, 1997; Sprenger *et al.*, 1997; Sévenier *et al.*, 1998
	Maize (fructan↑)	Caimi *et al.*, 1996
	Potato (fructan↑)	Hellwege *et al.*, 1997
	Sugar beet (fructan↑)	Smeekens, 1997
Fructose, raffinose, stachyose	Soybean	Hartwig *et al.*, 1997
Inulin	Potato (inulin↑)	Hellwege *et al.*, 2000
Starch	Rice (amylase↑)	Chiang *et al.*, 2005; Schwall, 2000
Micronutrients and functional metabolites		
Vitamins and carotenoids	Canola (vitamin E↑)	Shintani and DellaPenna, 1998
	Maize (vitamin E↑; vitamin C↑)	Rocheford *et al.*, 2002; Cahoon *et al.*, 2003; Chen *et al.*, 2003
	Mustard (+β-carotene)	Shewmaker et al., 1999
	Potato (β-carotene and lutein↑)	Ducreux *et al.*, 2005
	Rice (+β-carotene)	Ye *et al.*, 2000
	Strawberry (vitamin C↑)	Agius *et al.*, 2003
	Tomato (folate↑; phytoene and β-carotene↑; lycopene↑; provitamin A↑)	Rosati, 2000; Fraser *et al.*, 2001; Mehta *et al.*, 2002; Díaz de la Garza *et al.*, 2004; Enfissi *et al.*, 2005; DellaPenna, 2007

Table 1d. More examples of crops in research and/or development with nutritionally improved traits intended to provide health benefits for consumers and animals.

Traits	Crop (trait detail)	Reference
Functional secondary metabolites	Apple (+stilbenes)	Szanowski *et al.*, 2003
	Alfalfa (+resveratrol)	Hipskind and Paiva, 2000
	Kiwi (+resveratrol)	Kobayashi *et al.*, 2000
	Maize (flavonoids↑)	Yu et al., 2000
	Potato (anthocyanin and alkaloid glycoside↓; solanine↓)	Lukaszewicz *et al.*, 2004
	Rice (flavonoids↑; +resveratrol)	Stark-Lorenzen, 1997; Shin *et al.*, 2006
	Soybean (flavonoids↑)	Yu *et al.*, 2003
	Tomato (+resveratrol; chlorogenic acid↑; flavonoids↑; stilbene↑anthocynanins↑)	Rosati, 2000; Muir *et al.*, 2001; Niggeweg *et al.*, 2004; Giovinazzo *et al.*, 2005; Gonzali *et al.*, 2009
	Wheat (caffeic and ferulic acids↑; +resveratrol)	UPI, 2002
Mineral availability		
	Alfalfa (phytase↑)	Austin-Phillips *et al.*, 1999
	Lettuce (iron↑)	Goto *et al.*, 2000
	Rice (iron↑)	Lucca *et al.*, 2002
	Maize(phytase↑, ferritin↑)	Drakakaki, 2005; AllBusiness, 2009
	Soybean (phytase↑)	Denbow *et al.*, 1998
	Wheat (phytase↑)	Brinch-Pedersen *et al.*, 2000, 2006

From a health perspective, plant components of dietary interest can be divided into four main categories, which can be further broken down into positive and negative attributes for human nutrition, macronutrients [proteins, carbohydrates, lipids (oils), and fiber], micronutrients (vitamins, minerals, phytochemicals), anti-nutrients (substances such as phytate that limit bioavailability of nutrients), allergens, intolerances and toxins.

Technological Challenges

There are approximately 25,000 metabolites (phytochemicals)—of the 200,000 or so produced by plants—with known effects in the human diet (Go *et al.*, 2005). Analysis of these metabolites (most specifically metabolomic analysis) is a valuable tool in gaining better understanding of what has occurred during crop domestication (lost and silenced traits) and in designing new paradigms for more targeted crop improvement that is better tailored to current needs (Hall *et al.*, 2008). In addition, with modern techniques, we have the potential to seek out, analyze and introgress traits of value that were limited in previous breeding strategies. Research to improve the nutritional quality of plants has historically been limited by a lack of basic knowledge of plant metabolism and the challenge of resolving complex interactions of thousands of metabolic pathways. A complementarity of techniques, both traditional and novel, is needed to metabolically engineer plants to produce desired quality traits. Metabolic engineering is generally defined as the redirection of one or more reactions (enzymatic and otherwise) to improve the production of existing compounds, produce new compounds or mediate the degradation of undesirable compounds. It involves the redirection of cellular activities by the modification of the enzymatic, transport, and/or regulatory functions of the cell. Significant progress has been made in recent years in the molecular dissection of many plant pathways and in the use of cloned genes to engineer plant metabolism.

Although progress in dissecting metabolic pathways and our ability to manipulate gene expression in genetically modified (GM) plants has progressed apace, attempts to use these tools to engineer plant metabolism have not quite kept pace. Since the success of this approach hinges on the ability to change host metabolism, its continued development will depend critically on a far more sophisticated knowledge of plant metabolism—especially the nuances of interconnected cellular networks—than currently exists. This complex interconnectivity is regularly demonstrated. Relatively minor genomic changes (point mutations, single-gene insertions) are regularly observed following metabolomic analysis, to lead to significant changes in biochemical composition (Bino *et al.*, 2005; Davidovich-Rikanati *et al.*, 2007; Long *et al.*, 2006). Giliberto *et al.* (2005) used a genetic modification approach to study the mechanism of light influence on antioxidant content (anthocyanin, lycopene) in the tomato cultivar Moneymaker. However, other, what on the surface would appear to be more significant, genetic changes unexpectedly yield little phenotypical effect (Schauer and Fernie, 2006).

Likewise, unexpected outcomes are often observed. For example, significant modifications made to primary Calvin-cycle enzymes (fructose-1,6-bisphosphatase and phosphoribulokinase) have little effect while modifications to minor enzymes (*e.g.* aldase, which catalyzes a reversible reaction) seemingly irrelevant to pathway flux, have major

TABLE 2A. EXAMPLES OF PLANT COMPONENTS WITH SUGGESTED FUNCTIONALITY.[a]

Class/components	**Source**[b]	**Potential health benefit**
Carotenoids		
α-carotene	Carrots	Neutralizes free radicals that may cause damage to cells.
β-carotene	Various fruits, vegetables	Neutralizes free radicals.
Lutein	Green vegetables	Contributes to maintenance of healthy vision.
Lycopene	Tomatoes and tomato products (ketchup, sauces)	May reduce risk of prostate cancer.
Zeaxanthin	Eggs, citrus, maize	Contributes to maintenance of healthy vision.
Dietary fiber		
Insoluble fiber	Wheat bran	May reduce risk of breast and/or colon cancer.
Beta glucan	Oats	May reduce risk of cardiovascular disease (CVD).
Soluble fiber	Psyllium	May reduce risk of CVD.
Whole grains	Cereal grains	May reduce risk of CVD.
Collagen hydrolysate	Gelatin	May help improve some symptoms associated with osteoarthritis.
Fatty Acids		
Omega-3 fatty acids - DHA/EPA	Tuna; fish and marine oils	May reduce risk of CVD and improve mental, visual functions.
Conjugated linoleic acid (CLA)	Cheese, meat products	May improve body composition, may decrease risk of certain cancers.
Gamma linolenic Acid	Borage, evening primrose	May reduce inflammation risk of cancer, CVD disease and improve body composition.
Flavonoids		
Anthocyanidins: cyanidin	Berries	Neutralize free radicals, may reduce risk of cancer.
Hydroxycinnamates	Wheat	Antioxidant-like activities, may reduce risk of degenerative diseases.
Flavanols: catechins, tannins	Tea (green, catechins), (black, tannins)	Neutralize free radicals, may reduce risk of cancer.
Flavanones	Citrus	Neutralize free radicals, may reduce risk of cancer.
Flavones: quercetin	Fruits/vegetables	Neutralize free radicals, may reduce risk of cancer.

[a] Not an all-inclusive list.

[b] US Food and Drug Administration approved health claim established for component; modified from ILSI (2004a, b).

Table 2b. More examples of plant components with suggested functionality.

Class/components	Source	Potential health benefit
Glucosinolates, indoles, isothiocyanates		
Sulphoraphane	Cruciferous vegetables (broccoli, kale), horseradish	Neutralizes free radicals, may reduce risk of cancer.
Phenolics		
Stilbenes-resveratrol,	Grapes	May reduce risk of degenerative diseases; heart disease; cancer. May have longevity effect.
Caffeic acid, ferulic acid	Fruits, vegetables, citrus	Antioxidant-like activities; may reduce risk of degenerative diseases; heart disease, eye disease.
Epicatechin	Cacao	Antioxidant-like activities; may reduce risk of degenerative diseases; heart disease.
Plant stanols/sterols		
Stanol/sterol ester	Maize, soy, wheat, wood oils	May reduce risk of coronary heart disease (CHD) by lowering blood cholesterol levels.
Prebiotics/probiotics		
Fructans, inulins, fructo-oligosaccharides (FOS)	Jerusalem artichokes, shallots, onion powder	May improve gastrointestinal health.
Lactobacillus	Yogurt, other dairy	May improve gastrointestinal health.
Saponins	Soybeans, soy foods, soy protein-containing foods	May lower LDL cholesterol; contains anti-cancer enzymes.
Soybean protein	Soybeans and soy-based foods	25 g/day may reduce risk of heart disease.
Phytoestrogens		
Isoflavones - daidzein, genistein	Soybeans and soy-based foods	May reduce menopause symptoms, such as hot flashes, reduce osteoporosis, CVD.
Lignans	Flax, rye, vegetables	May protect against heart disease and some cancers; may lower LDL cholesterol, cholesterol, total cholesterol, and triglycerides.
Sulfides/thiols		
Diallyl sulfide	Onions, garlic, olives, leeks, scallions	May lower LDL cholesterol, helps to maintain healthy immune system.
Allyl methyl trisulfide, dithiolthiones	Cruciferous vegetables	May lower LDL cholesterol, helps to maintain healthy immune system.
Tannins		
Proanthocyanidins	Cranberries, cranberry products, cocoa, chocolate, black tea	May improve urinary tract health. May reduce risk of CVD, and high blood pressure.

effects (Paul *et al.*, 1995; Hajirezaei *et al.*, 1994). These observations demonstrate that caution must be exercised when extrapolating individual enzyme kinetics to the control of flux in complex metabolic pathways. With evolving "omics" tools, a better understanding of global effects of metabolic engineering on metabolites, enzyme activities, and fluxes is beginning to be developed. Attempts to modify storage proteins or secondary metabolic pathways have also been more successful than have alterations of primary and intermediary metabolism (DellaPenna and Pogson, 2006). While offering many opportunities, this plasticity in metabolism complicates potential routes to the design of new, improved crop varieties. Regulatory oversight of engineered products has been designed to detect such unexpected outcomes in biotech crops and, as demonstrated by Chassy *et al.* (ILSI, 2004a, b, 2008), existing analytical and regulatory systems are adequate to address novel metabolic modifications in nutritionally improved crops.

A number of new approaches are being developed to counter some of the complex problems in metabolic engineering of pathways. Such approaches include use of RNA interference to modulate endogenous gene expression or the manipulation of transcription factors (TFs) that control networks of metabolism (Kinney, 1998; Bruce *et al.*, 2000; Butelli *et al.*, 2009, Gonzali *et al.*, 2009). For example, expression in tomato of two selected TFs involved in anthocyanin production in snapdragon (*Antirrhinum majus* L.) led to high levels of these flavonoids throughout the fruit tissues, which, as a consequence, were purple. They also stimulated genes involved in the side-chain modification of the anthocyanin pigments and genes possibly related to the final transport of these molecules into the vacuole, processes that are both necessary for the accumulation of anthocyanin (Gonzali *et al.*, 2009). Such expression experiments hold promise as an effective tool for the determination of transcriptional regulatory networks for important biochemical pathways. Gene expression can be modulated by numerous transcriptional and posttranscriptional processes. Correctly choreographing the many variables is the factor that makes metabolic engineering in plants so challenging.

In addition, there are several new technologies that can overcome the limitation of single-gene transfers and facilitate the concomitant transfer of multiple components of metabolic pathways. One example is multiple-transgene direct-DNA transfer, which simultaneously introduces all the components required for the expression of complex recombinant macromolecules into the plant genome as demonstrated by a number of reports, including those of Nicholson *et al.* (2005), who successfully delivered four transgenes that represent the components of a secretory antibody into rice, and of Carlson *et al.* (2007) who constructed a minichromosome vector that remains autonomous from the plant's chromosomes and stably replicates when introduced into maize cells. This work makes it possible to design minichromosomes that carry cassettes of genes, enhancing the ability to engineer plant processes such as the production of complex biochemicals. Christou and Kohli (2009) demonstrated that gene transfer using minimal cassettes is an efficient and rapid method for the production of transgenic plants stably expressing several different transgenes. Since no vector backbones are required, this prevents the integration of potentially recombinogenic sequences insuring stability across generations. They used combinatorial direct-DNA transformation to introduce multi-complex metabolic pathways synthesizing β-carotene, vitamin C and folate. They achieved this by transferring five constructs controlled by various endosperm-specific promoters into white maize. Different enzyme combinations show distinct metabolic phenotypes resulting in a 169-fold increase in β-carotene, a five-fold increase vitamin C, and a doubling in folate production, effectively creating a multivitamin maize cultivar (Naqvi *et al.*, 2009). This system has an added advantage from a commercial perspective in that these methods circumvent problems with traditional approaches that not only limit the amount of sequences transferred, but may disrupt native genes or lead to poor expression of the transgene, thus reducing both the numbers of transgenic plants that must be screened and the subsequent breeding and introgression steps required to select a suitable commercial candidate.

As demonstrated, "omics"-based strategies for gene and metabolite discovery, coupled with high-throughput transformation processes and automated analytical and functionality assays, have accelerated the identification of product candidates. Identifying rate-limiting steps in synthesis could provide targets for modifying pathways for novel or customized traits. Targeted expression will be used to channel metabolic flow into new pathways, while gene-silencing tools will reduce or eliminate undesirable compounds or traits, or switch off genes to increase desirable products (Liu *et al.*, 2002; Herman *et al.*, 2003; Davies, 2007). In addition, molecular marker-based breeding strategies have already been used to accelerate the process of introgressing trait genes into high-yielding germplasm for commercialization. Tables 1a–d summarize the work done to date on specific applications in the categories listed above. The following sections briefly review some examples under those categories.

MACRONUTRIENTS

Protein

The FAO estimates that 850 million people worldwide suffer from under-nutrition, of which insufficient protein in the diet is a significant contributing factor (FAO, 2004). Protein-energy malnutrition (PEM) is the most lethal form of malnutrition and affects every fourth child worldwide according to the WHO (2006). Most plants have a poor balance of essential amino acids relative to the needs of animals and humans. The cereals (maize, wheat, rice, *etc.*) tend to be low in lysine, whereas legumes (soybean, pea, *etc.*) are often deficient in the sulfur-rich amino acids, methionine and cysteine. Successful examples of improving amino acid balance to date include high-lysine maize (Eggeling *et al.*, 1998; O'Quinn *et al.*, 2000) canola and soybean (Falco *et al.*, 1995). Free lysine is significantly increased in high-lysine maize by the introduction of the dapA gene (cordapA) from *Corynebacterium glutamicum*, which encodes a form of dihydrodipicolinate synthase (cDHDPS) that is insensitive to lysine feedback inhibition. Consumption of foods made from these crops potentially can help to prevent malnutrition in developing countries, especially among children.

Another method of modifying storage-protein composition is to introduce heterologous or homologous genes that code for proteins containing elevated levels of the desired amino acid, such as sulfur-containing methionine and cysteine, or lysine. An interesting solution to this is to create a completely artificial protein containing the optimum number of the essential amino acids—methionine, threonine, lysine, isoleucine and leucine—in a stable, helical conformation designed to resist proteases to prevent degradation. This has been achieved by a number of investigators, including a sweet potato modified with an artificial storage protein (ASP-1) gene (Prakash *et al.*, 2000). These transgenic plants exhibited two- and five-fold increases in the total protein contents in leaves and roots, respectively, over those of control plants. Significant increases in the levels of essential amino acids were also observed (Prakash *et al.*, 2000; ILSI, 2008). A key issue is to ensure that total amount and composition of storage proteins is not altered to the detriment of the development of the crop plant when attempting to improve amino-acid ratios (Rapp *et al.*, 2002).

Some novel indirect approaches have also been taken to improve protein content. Uauy *et al.* (2006) "rescued" an ancestral wheat allele that encodes a transcription factor (NAM-B1), which accelerates senescence and increases nutrient remobilization from leaves to developing grains (modern wheat varieties carry a nonfunctional allele.) Reduction in RNA levels of the multiple NAM homologs by RNA interference delayed senescence by more than 3 weeks and reduced wheat-grain protein, zinc, and iron contents by more than 30%. Young *et al.* (2004) used yet another approach to indirectly increase protein and oil content. They used a bacterial cytokinin-synthesizing isopentenyl transferase (IPT) enzyme, under the control of a self-limiting senescence-inducible promoter, to block the loss of the lower floret resulting in the production of just one kernel composed of a fused endosperm with two viable embryos. The presence of two embryos in a normal-sized kernel leads to displacement of endosperm growth, resulting in kernels with an increased ratio of embryo-to-endosperm content. The end result is maize with more protein and oil and less carbohydrate (Young *et al.*, 2004; ILSI, 2008).

Fiber and Carbohydrates

Fiber is a group of substances chemically similar to carbohydrates that non-ruminant animals, including humans, poorly metabolize for energy or other nutritional uses. Fiber provides bulk in the diet such that foods rich in fiber offer satiety without contributing significant calories. Current controversies aside, there is ample scientific evidence to show that prolonged intake of dietary fiber has various positive health benefits, especially the potential for reduced risk of colon and other types of cancer.

Recent microbiome twin studies by Jeff Gordon addressing the interrelationships between diet and gut microbial community structure/function indicated that differences in our gut microbial ecology affect our pre-disposition to obesity or malnutrition and that diet rather than applied probiotics was the single most important characterization of gut health (Turnbaugh *et al.*, 2009). These studies involved characterization of the gut microbiota/microbiome of twins, concordant or discordant for malnutrition, living in several developing countries, who were sampled just prior to, during and after treatment.

When such colonic bacteria (especially bifidobacteria) ferment dietary fiber or other unabsorbed carbohydrates, the products are short-chain saturated fatty acids. These short-chain fatty acids may enhance absorption of minerals such as iron, calcium, and zinc, induce apoptosis—preventing colon cancer—and inhibit 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoAR), thus lowering low-density lipoprotein (LDL) production (German, 2005). Plants are effective at making both polymeric carbohydrates (*e.g.* starches and fructans), and individual sugars (*e.g.* sucrose and fructose). The biosynthesis of these compounds is sufficiently understood to allow the bioengineering of their properties and to engineer crops to produce polysaccharides not normally present. Polymeric carbohydrates such as fructans have been produced in sugar beet, and inulins and amylase (resistant starch) in potato (Hellwege *et al.* (2000) without adverse affects on growth or phenotype. A similar approach is being used to derive soybean varieties that contain some oligofructan components that selectively increase the population of beneficial species of bacteria in the intestines of humans and certain animals and inhibit growth of harmful ones (Bouhnik *et al.*, 1999).

Novel Lipids

Genomics, specifically marker-assisted plant breeding, combined with recombinant DNA technology provide powerful means for modifying the composition of oilseeds to improve their nutritional value and provide the functional properties required for various food-oil applications. Genetic modification of oilseed crops can provide an abundant, relatively inexpensive source of dietary fatty acids with wide-ranging health benefits. Production of such lipids in vegetable oil provides a convenient mechanism to deliver healthier products to consumers without the requirement for significant dietary changes. Major alterations in the proportions of individual fatty acids have been achieved in a range of oilseeds using conventional selection, induced mutation and, more recently, post-transcriptional gene silencing. Examples of such modified oils include: low- and zero-saturated fat soybean and canola oils, canola oil containing medium chain fatty acids (MCFA) whose ergogenic potential may have application in LDCs, high stearic acid canola oil (for *trans* fatty acid-free products), high oleic acid (monounsaturated) soybean oil, and canola oil containing the polyunsaturated fatty acids (PUFA), λ-linolenic (GLA; 18:3 n-6) stearidonic acids (SDA; C18:4 n-3), very-long-chain fatty acids (Zou *et al.*, 1997) and ω-three fatty acids (Yuan and Knauf, 1997). These modified oils are being marketed and many countries have a regulatory system in place for the pre-market safety review of novel foods produced through conventional technology.

Edible oils rich in monounsaturated fatty acids provide improved oil stability, flavor, and nutrition for human and animal consumption. High-oleic soybean oil is naturally more resistant to degradation by heat and oxidation, and so requires little or no post-refining processing (hydrogenation), depending on the intended application. Oleic acid (18:1), a monounsaturate, can provide more stability than the polyunsaturates, linoleic (18:2) and linolenic (18:3). Antisense inhibition of oleate desaturase expression in soybean resulted in oil that contained >80% oleic acid (23% is normal) and had a significant decrease in PUFA (Kinney and Knowlton, 1998). Dupont has introduced soybean oil composed of at least 80% oleic acid, and linolenic acid of about 3%, and over 20% less saturated fatty acids than commodity soybean oil. Monsanto's Vistive contains less than 3% linolenic acid, compared to 8% for traditional soybeans, resulting in more stable soybean oil and less need for hydrogenation.

A key function of α-linolenic acid (ALA) is as a substrate for the synthesis of longer-chain ω-3 fatty acids found in fish, eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), which play an important role in the regulation of inflammatory immune reactions and blood pressure, brain development *in utero*, and, in early postnatal life, the development of cognitive function. Stearidonic acid, EPA, and DHA also possess anti-cancer properties (Christensen *et al.*, 1999; Smuts *et al.*, 2003; Reiffel and McDonald, 2006). Research indicates that the ratio of n-3 to n-6 fatty acids may be as important to health and nutrition as the absolute amounts present in the diet or in body tissues. Current western diets tend to be relatively high in n-6 fatty acids and relatively low in n-3 fatty acids. Production of a readily available source of long-chain-PUFA, specifically ω-3 fatty acids, delivered in widely consumed prepared foods could deliver much needed ω-3-fatty acids to large sectors of the population with skewed n-6: n-3 ratios. In plants, the microsomal ω-6 desaturase-catalyzed pathway is the primary route of production of polyunsaturated lipids. Ursin *et al.* (2000, 2003) has introduced the Δ-6 desaturase gene from a fungus (*Mortierella*) succeeding in producing ω-3 in canola. In a clinical study, James *et al.* (2003) observed that SDA was superior to ALA as a precursor by a factor of 3.6 in producing EPA, DHA and docosapentaenoic acid (DPA, C22:5n-3). Transgenic canola oil was obtained that contains >23% SDA, with an overall n-6: n-3 ratio of 0.5.

However, not all ω-6 FAs are created equal. Gamma linolenic acid (GLA, C18:3n-6) is an ω-6 fatty acid with health benefits that are similar and complementary to the benefits of ω-3 FAs including anti-inflammatory effects, improved skin health and weight-loss maintenance (Schirmer and Phinney, 2007). A Davis, CA, company, Arcadia, has engineered GLA safflower oil, with up to 40% GLA, essentially quadrupling the levels obtained in source plants such as evening primrose and borage (BNET, 2008). Structural lipids also have positive health benefits; for example, in addition to their effect in lowering cholesterol, membrane lipid phytosterols have been found to inhibit the proliferation of cancer cells by inducing apoptosis and G1/S cell-cycle arrest through the HMG-CoAR as noted above (Awad, 2000). In addition to this and the above, specialty oils may also be developed with further pharmaceutical and chemical feedstock applications in mind.

MICRONUTRIENTS

Vitamins and Minerals

Micronutrient malnutrition, the so-called hidden hunger, affects more than half of the world's population, especially women and preschool children in developing countries (UN SCN, 2004). Even mild levels of micronutrient malnutrition may damage cognitive development and lower disease resistance in children, and increase incidence of childbirth mortality. The costs of these deficiencies, in terms of diminished quality of life and lives lost, are large (Pfeiffera and McClafferty, 2007). The clinical and epidemiological evidence is clear that select minerals (iron, calcium, selenium and iodine) and a limited number of vitamins (folate, vitamins E, B6 and A) play significant roles in maintenance of optimal health and are limiting in diets.

As with macronutrients, one way to ensure an adequate dietary intake of nutritionally beneficial phytochemicals is to adjust their levels in plant foods. Using various approaches including genomics, vitamin-E levels are being increased in several crops, including soybean, maize and canola, while rice varieties are being developed with the enhanced vitamin-A precursor, β-carotene, to address vitamin-A deficiency that leads to macular degeneration and impacts development. A similar method was used by Monsanto to produce β-carotene in canola and by Fauquet in cassava. The latter is being field tested in Nigeria. Ameliorating another major deficiency in LDCs, namely of minerals such as iron and zinc, has also been addressed. Iron is the most commonly deficient micronutrient in the human diet, affecting an estimated 1 to 2 billion people. Anemia, characterized by low hemoglobin, is the most widely recognized symptom of iron deficiency, but there are other serious problems such as impaired learning ability in children, increased susceptibility to infection, and reduced work capacity. Drakakaki *et al.* (2005) demonstrated endosperm-specific co-expression of recombinant soybean ferritin and *Aspergillus* phytase in maize which resulted in significant increases in the levels of bioavailable iron. A similar end was achieved with lettuce (Goto *et al.*, 2000).

A rather interesting approach was taken by Connolly (2008) to increase the levels of calcium in crop plants by using a modified calcium/proton antiporter [known as short cation exchanger 1 (sCAX1)] to increase Ca transport into vacuoles. She also demonstrated that consumption of such Ca-fortified carrots results in enhanced Ca absorption. This demonstrates the potential of increasing plant-nutrient content through expression of a high-capacity transporter and illustrates the importance of demonstrating that the fortified nutrient is bioavailable. Other targets include folate-enriched tomatoes and isoflavonoids (DellaPenna, 2007; Yonekura-Sakakibara *et al.*, 2007).

MICRONUTRIENTS

Phytochemicals

Unlike for vitamins and minerals, the primary evidence for the health-promoting roles of phytochemicals comes from epidemiological studies, and the exact chemical identities of many active compounds have yet to be determined. However, for select groups of phytochemicals, such as non-provitamin-A carotenoids, glucosinolates, and phytoestrogens, the active compound or compounds have been identified and rigorously studied. Epidemiologic studies have suggested a potential benefit of the carotenoid lycopene in reducing the risk of prostate cancer, particularly the more lethal forms of this cancer. Five studies support a 30% to 40% reduction in risk associated with high tomato or lycopene consumption in the processed form in conjunction with lipid consumption, although other studies with raw tomatoes were not conclusive (Giovannucci, 2002). Since carotenoids are lipid soluble and cooking breaks down carotenoid-binding proteins, this is not an unexpected outcome. In a study by Mehta *et al.* (2002) to modify polyamines to retard tomato ripening, they found an unanticipated enrichment in lycopene with levels up by 2- to 3.5-fold compared to conventional tomatoes (Table 1c). This is a substantial enrichment, exceeding that so far achieved by conventional means. This approach may work in other fruits and vegetables. Flavonoids, meanwhile, are soluble in water, and foods containing both water soluble and fat-dissolved antioxidants are considered to offer the best protection against disease. Anthocyanins offer protection against certain cancers, cardiovascular disease and age-related degenerative diseases. There is evidence that anthocyanins also have anti-inflammatory activity, promote visual acuity and hinder obesity and diabetes. Gonzali *et al.* (2009) and Butelli *et al.* (2008) used snapdragon-transcription factors to achieve high levels of expression of oxygen-scavenging anthocyanins in tomatoes. In a pilot test, the lifespan of cancer-susceptible mice was significantly extended when their diet was supplemented with purple tomatoes compared to supplementation with normal red tomatoes.

Other phytochemicals of interest include related polyphenolics such as resveratrol, which has been demonstrated to inhibit platelet aggregation and eicosanoid synthesis in addition to protecting the sirtuins, genes implicated in DNA modification and life extension; flavonoids, such as tomatoes expressing chalcone isomerase that show increased contents of the flavanols rutin and kaempferol glycoside; glucosinolates and their related products such as indole-3 carbinol (I3C); catechin and catechol; isoflavones, such as genistein and daidzein; anthocyanins; and some phytoalexins (Table 1d). A comprehensive list of phytochemicals is provided in Table 2. To reiterate: although a growing knowledge base indicates that elevated intakes of specific phytochemicals may reduce the risk of diseases, such as certain cancers, cardiovascular diseases, and chronic degenerative diseases associated with aging, further research and epidemiological studies are still required to prove definitive relationships.

Plants Fighting Back

Plants produce many defense strategies to protect themselves from predators. Many, such as resveratrol and glucosinolates, which are primarily pathogen-protective chemicals, also have demonstrated beneficial effects for human and animal health. Many, however, have the opposite effect. For example, phytate, a plant phosphate-storage compound, is considered an anti-nutrient as it strongly chelates iron, calcium, zinc and other divalent mineral ions, making them unavailable for uptake. Non-ruminant animals generally lack the phytase enzyme needed for digestion of phytate. Poultry and swine producers add processed phosphate to their feed rations to counter this. Excess phosphate is excreted into the environment resulting in water pollution. When low-phytate soybean meal is utilized along with low-phytate maize for animal feeds, the phosphate excretion in swine and poultry manure is halved. A number of groups have added heat-and acid-stable phytase from *Aspergillus fumigatus inter alia* to make the phosphate and liberated ions bioavailable in several crops (Potrykus, 1999). To promote the reabsorption of iron, a gene for a metallothionein-like protein has also been engineered. Low-phytate maize was commercialized in the United States in 1999 (Wehrspann, 1998). In November 2009, the Chinese company Origin Agritech announced the final approval of the world's first genetically modified phytase-expressing maize (AllBusiness, 2009). Research indicates that the protein in low-phytate soybean is also slightly more digestible than the protein in traditional soybean. In a poultry-feeding trial, better results were obtained using transgenic plant material than with the commercially produced phytase supplement (Keshavarz, 2003). Poultry grew well on the engineered alfalfa diet without any inorganic phosphorus supplement, which shows that plants can be tailored to increase the bioavailability of this essential mineral. A similar effect was achieved in wheat by a Danish group, whose temperature-tolerant phytase resisted boiling (Brinch-Pedersen *et al.*, 2006)

Other anti-nutrients that are being examined as possible targets for reduction are trypsin inhibitors, lectins, and several heat-stable components found in soybean and other crops. Likewise, strategies are being employed to reduce or limit food allergens (albumins, globulins, *etc.*), malabsorption and food intolerances (gluten) and toxins (glycoalkaloids, cyanogenic glucosides, phytohemagglutinins) in crop plants and aesthetic undesirables such as caffeine (Ogita, 2003). Examples include changing the levels of expression of the thioredoxin gene to reduce the intolerance effects of wheat and other cereals (Buchanan *et al.*, 1997). Using RNAi to silence the major allergen in soybean (P34 a member of the papain superfamily of cysteine proteases) and rice (14–16 kDa allergenic proteins). Blood-serum tests indicate that p34-specific IgE antibodies could not be detected after consumption of gene-silenced beans (Helm *et al.*, 2000; Herman *et al.*, 2003).

Modern biotech approaches can be employed to down-regulate or even eliminate the genes involved in the metabolic pathways for the production, accumulation, and/or activation of toxins in plants. For example, the solanine content of potato has already been reduced substantially using an antisense approach, and efforts are underway to reduce the level of the other major potato glycoalkaloid, chaconine (McCue *et al.*, 2003). Work has also been done to reduce cyanogenic glycosides in cassava through expression of the cassava enzyme hydroxynitrile lyase in the roots (Siritunga and Sayre, 2003).

When "disarming" plants' natural defenses in this way, one must be aware of potentially increased susceptibility to pests, diseases and other stressors, therefore the recipient germplasm should have input traits to counter this.

Prospects for Crop Biotechnology

Improvement of crop-nutritional quality is a technical challenge hampered by a lack of basic knowledge of plant metabolism and the need to resolve the complexity of intersecting networks of thousands of metabolic pathways. With the tools now available through the field of genomics, proteomics, lipomics, glycobiomics, metabolomics and bioinformatics, we have the potential to study and manipulate genes and pathways at the metalevel, and simultaneously study the expression and interaction of transgenes on tens of thousands of endogenous genes. With these newly evolving tools, we are beginning to dissect the global effects of metabolic engineering on metabolites, enzyme activities and fluxes. For essential macro- and micronutrients that are limiting in various regional diets, the strategies for improvement are clear and concerns such as pleiotropic effects and safe upper limits are easily addressed. However, for many putative health-promoting phytochemicals, clear links with health benefits are yet to be demonstrated. In addition, one must be careful when extrapolating attributes from an individual substance acting independently to that substance acting within a complex milieu. However, if such links can be established, it will make it possible to identify the precise compound or compounds to target and which crops to modify to achieve the greatest nutritional impact and health benefit. With rapidly emerging technologies, the increase in our understanding of, and ability to manipulate, plant metabolism during the coming decades should place plant researchers in the position of being able to modify the nutritional content of major and minor crops to improve many aspects of human and animal health and wellbeing.

However, the actual commercialization of such products may have little to do with technical limitations and more to do with external constraints, primarily the process of regulatory approval. The flagship of improved nutritional varieties, namely β-carotene-enhanced rice commonly referred to as "golden rice," despite being under consideration since the late nineties and subject to a barrage of risk assessments, is unlikely to be approved until 2012 at the earliest. Ingo Potrykus, the developer, says that an unreasonable amount of testing has been required without scientific justification. In a recent *Nature* article (Potrykus, 2010) he laid the blame solely at the door of the regulatory process, which he considers excessive, observing that unjustified and impractical legal requirements are stopping genetically engineered crops from saving millions from starvation and malnutrition.

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