thereby reducing the breeding requirements for transgene introductions. The TopCross system also provides a way to produce traits that have a negative effect on seed agronomic performance, such as on germination, but are desirable in the grain, as the trait can be introduced solely through the pollinator.

To date, a number of output traits have been developed at DuPont that are in various stages of functional testing or commercialization. These include corn and soybean lines for animal feed applications with increased concentrations of free lysine (*7*) or bound methionine (*31*). Soybean seeds with altered ratios of seed storage proteins (*26*) or reduced concentrations of trypsin inhibitors (*32*) have been produced, primarily for food applications. As described earlier, soybean lines with novel fatty acid compositions have been developed. High monounsaturate oils are useful in foods or food-processing applications where increased oxidative and thermal stability is desirable, and high saturate oils are useful as feedstocks for margarine manufacturing. All of these oils also have nutritional or health benefits. The soybean lines with reduced oligosaccharide concentrations described above and similar corn lines contain reduced concentrations of phytic acid and increased concentrations of free phosphorous. This combination provides nutritional value in animal feed and environmental value because of reduced bound phosphorous released in animal waste. Finally, a number of starches have been created for use in food and industrial applications, including polymers with altered branching patterns, altered branch lengths, and novel monomer compositions (*33*).

Plants are capable of a stunning array of biochemical conversions. As an understanding of the regulation of these pathways is combined with the ability to engineer multiple genes simultaneously, the possibilities for producing novel products in plants will increase dramatically. Beyond the traditional agricultural products, opportunities exist for producing industrial feedstocks and polymers in crops and for producing pharmaceutical and nutraceutical products. Traits that have been initially produced in corn or soybeans may enter new markets through production in cereals such as wheat and rice or in plantation crops such as forest trees. The continuing integration of structural and functional genomics with trait development technologies will accelerate these advances.

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REVIEW

Nutritional Genomics: Manipulating Plant Micronutrients to Improve Human Health

Dean DellaPenna

The nutritional health and well-being of humans are entirely dependent on plant foods either directly or indirectly when plants are consumed by animals. Plant foods provide almost all essential vitamins and minerals and a number of other health-promoting phytochemicals. Because micronutrient concentrations are often low in staple crops, research is under way to understand and manipulate synthesis of micronutrients in order to improve crop nutritional quality. Genome sequencing projects are providing novel approaches for identifying plant biosynthetic genes of nutritional importance. The term "nutritional genomics" is used to describe work at the interface of plant biochemistry, genomics, and human nutrition.

Humans require a diverse, well-balanced diet containing a complex mixture of both macronutrients and micronutrients in order to maintain optimal health. Macronutrients—carbohydrates, lipids, and proteins (amino acids)—make up the bulk of foodstuff and are used primarily as an energy supply. Micronutrients are organic or inorganic compounds present in small amounts and are not used for energy, but are nonetheless needed for good health. Essential micronutrients in the human diet include 17 minerals and 13 vitamins required at minimum levels to alleviate nutritional disorders (Table 1). Nonessential micronutrients encompass a vast group of unique organic phytochemicals that are not strictly required in the diet, but when present at sufficient levels are linked to the promotion of good health.

Modifying the nutritional composition of plant foods is an urgent worldwide health issue as basic nutritional needs for much of the world's population are still unmet. Large numbers of people in developing countries exist on simple diets composed primarily of a few staple foods (cassava, wheat, rice, and corn) that are poor sources of some macronutrients and many essential micronutrients. Consequently, the diet of over 800 million people does not contain sufficient macronutrients, and micronutrient deficiencies are even more prevalent (*1*). As examples of the magnitude of micronutrient deficiencies, estimates place 250 million children at risk for

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vitamin A deficiency (in up to 500,000 annually, this deficiency will cause irreversible blindness), 2 billion people at risk for iron deficiency (with children and women of reproductive age particularly vulnerable), and 1.5 billion people at risk for iodine deficiency (*2*). Even in industrialized nations, where both food abundance and variety are excellent and daily caloric intake is often excessive, micronutrient deficiencies are surprisingly common owing to poor eating habits.

While a plant-based diet can, in theory, ensure the adequate nutrition of humans at all stages of life, in practice, plant micronutrient levels vary widely and dietary micronutrient intake varies depending on the primary plant food source. Major staple crops contain insufficient concentrations of many essential vitamins and minerals to meet the U.S. recommended dietary allowance (RDA); therefore, nutrient fortification of the food supply is a necessary practice (*3*). RDAs can also be somewhat misleading because they are not the levels needed for optimal health, but rather the minimum levels needed to alleviate specific nutritional disorders (*4*). As such, RDAs do not reflect the growing knowledge base indicating that the elevated intake of specific vitamins and minerals (for example, vitamins E and C, carotenoids, and selenium) significantly reduces the risk of diseases such as certain cancers, cardiovascular diseases, and chronic degenerative diseases associated with aging (*5–7*). In order to obtain such therapeutic levels in the diet, additional fortification of the food supply will be required as well as modification of dietary preferences, or direct modification of micronutrient levels in food crops.

Table 1. Selected essential micronutrients in the human diet, their daily requirements, and safe upper intake levels.

Nutrient	Maximum adult RDA*	Safe upper intake (relative to RDA)†
	Minerals	
Calcium (*31*)	1200 mg	2×
Iron (*32*)	15 mg	5×
Iodine (*33*)	150 μg	13×
Selenium (*34*)	70 μg	13×
	Water-soluble vitamins	
Vitamin C (*35*)	60 mg	16×
Vitamin B_6 (*36*)	2 mg	125×
Folate (*37*)	200 μg	50×
Biotin‡ (*38*)	30–100 μg	300×
	Fat-soluble vitamins	
Vitamin E (*39*)	10 mg α-TE§	100×
Vitamin A‖ (*40*)	1 mg RE¶	5× (retinol)
		100× (β-carotene)

*Recommended dietary allowances per day. Values presented are the highest RDA either for male or female adults, excluding pregnant or lactating women (*41*). †The safe upper intake limit, excluding pregnant or lactating women, is associated with a low probability of adverse side effects and assumes individual variation in requirements and tolerance to high levels (*10, 30, 41*). The author is not advocating intake of supplements at these levels. ‡The recommended value for biotin is provided as estimated safe and adequate daily dietary intake, because less information is available on which to base the RDA (*30*). §One α-TE (α -tocopherol equivalent) is equal to 1 mg of (*R,R,R*)-α-tocopherol. ‖Vitamin A (retinol) is not made in plants; however, plants contain a number of provitamin A carotenoids (for example, β-carotene) that can be metabolized to vitamin A. ¶ Vitamin A activity is expressed in retinol equivalents (RE). One RE is equal to 1 mg of all-*trans*-retinol, 6 mg of all-*trans*-β-carotene, or 12 mg of other provitamin A carotenoids.

In addition to essential vitamins and minerals, plants also synthesize 80,000 of the 100,000 characterized secondary metabolites on the planet (*8*). This myriad of phytochemicals can be separated into several groups, with some containing several thousand chemically distinct compounds (Table 2). Unlike the ubiquitous vitamins and minerals, specific phytochemicals are often unique to certain plant species or genera where they have evolved to play roles in development, stress responses, defense, or central and secondary metabolism. Many phytochemicals also have significant consequences for human health and are thought to be a major reason that plant-rich diets are associated with lower morbidity and mortality in adult life (*9*). Unfortunately, many of the best-characterized health-promoting phytochemicals are only present in plants or plant-derived products that are consumed at low levels in the American diet (Table 2). Thus, because of dietary preferences, the health benefits associated with intake of specific phytochemicals are not fully realized in most American populations.

Micronutrient Targets: General Considerations and Concerns

To ensure an adequate dietary intake of all essential vitamins and minerals and to increase the consumption of health-promoting phytochemicals, researchers have turned with renewed interest to the study and manipulation of plant secondary metabolism. A major focus is the identification and isolation of genes required for the synthesis and accumulation of a target compound such that its levels can be modified in staple crops to effect the desired dietary change. A key point for all micronutrient research is that unlike macronutrients, which can account for up to 30% of a tissue's dry weight, individual micronutrients are generally much less than 0.1% of a tissue's dry weight and thus significant increases in micronutrient levels are theoretically feasible.

Before attempting to manipulate nutritional components in food crops, careful consideration must be given to the selection of target compounds, their efficacy, and whether excessive dietary intake could have unintended negative health consequences. For select mineral targets (iron, calcium, selenium, and iodine) and a limited number of vitamin targets (folate, vitamins E, B_6, and A), the clinical and epidemiological evidence is clear that they play a significant role in maintenance of optimal health and are limiting in diets worldwide (*10*). The upper safe levels of intake for these minerals range from 2 to 13 times the RDA, while for these vitamins it is generally much higher, allowing a greater range for manipulation (Table 1). The notable vitamin exception is vitamin A (retinol), which can cause side effects at five times the RDA. Fortuitously, plants only synthesize provitamin A carotenoids, which are used as substrates for retinol synthesis by humans. The overall process is highly regulated and as a consequence, the upper safe intake level for β-carotene (the most active provitamin A carotenoid in plants) is 20 times that of retinol or 100 times the RDA for vitamin A. On this basis, prudence would dictate manipulating provitamin A carotenoid synthesis in plants, rather than attempting to introduce retinol synthesis. By following similar logic in selecting other compounds for manipulation, potentially negative health issues can be minimized or avoided entirely.

Unlike vitamins and minerals, the primary evidence for the health-promoting roles of phytochemicals comes from epidemiological studies (*7*), and the exact chemical identity of many active compounds has yet to be determined. However, for select groups of phytochemicals, such as non–provitamin A carotenoids, glucosinolates, and phytoestrogens (Table 2), the active compound or compounds have been identified and rigorously studied. Glucosinolates are present in cruciferous vegetables (broccoli is a good source) and target Phase 1 and Phase 2 drug-metabolizing enzymes in humans, leading to decreased carcinogen-DNA interactions and increased carcinogen detoxification (*11, 12*). The phytoestrogens, genistein and daidzein, are health-promoting isoflavones that are particularly abundant in soybeans. Studies have shown that individuals with soy-rich diets have significantly lower occurrences of some cancers, osteoporosis, and coronary heart disease when compared to individuals with low soy diets (*13*). Finally, dietary carotenoids (both pro- and non–provitamin A) have received considerable attention for reducing the risk of certain types of cancers, cardiovascular disease, and age-related macular degeneration (*5, 14*). Although glucosinolates, isoflavones, and carotenoids are clearly beneficial to human health, Western diets are generally poor in foods containing the highest levels, and a strong case for manipulating their synthesis in staple food crops can be made.

Manipulating Plant Micronutrient Content Through Plant Breeding

Before discussing the promise that emerging technologies hold for manipulating plant micronutrient content, it is important to stress that more traditional approaches exist and should be pursued both independently and in conjunction with emerging technologies. During the past 50 years, the primary objective of modern agriculture and breeding programs has been to increase productivity and yields, a quest that will remain a principal concern in providing the caloric intake needed by a growing world population. However, equally as important, but largely overlooked in breeding programs, is the micronutrient composition and density of crops. In the rare cases where micronutrient content has been assessed, significant genotypic variation has been observed (*15*, *16*). Such variation should and is being used to develop nutritionally improved cultivars and assist in identifying the genetic and physiological basis for nutrient variation (*17*).

Molecular Genetic Approaches to Dissecting Plant Secondary Metabolism

Given its enormous biochemical diversity, plant secondary metabolism presents special problems for researchers owing to difficulty in identifying, purifying, and assaying the enzymes involved. As a result, although radiotracer studies produced maps for much of plant secondary metabolism by the late 1970s, obtaining gene probes for target enzymes through classic biochemical approaches has been and continues to be a difficult and limiting step. Early efforts to manipulate secondary metabolism were also stymied by a lack of basic knowledge about mechanisms regulating the synthesis and accumulation of target compounds. These studies make it clear that only by increasing our global understanding of plant secondary metabolism can the manipulation of essential micronutrients and nonessential phytochemicals be achieved.

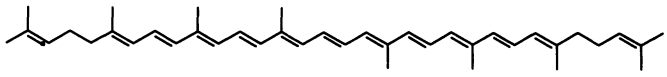
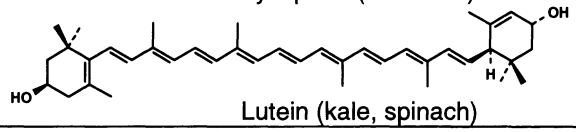
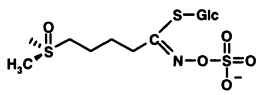
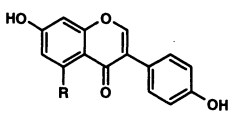
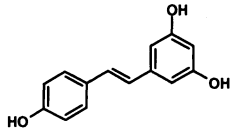
For many plant secondary metabolites, including those of nutritional importance to humans, gene identification by classical biochemical approaches has given way to molecular genetic approaches. For example, *Arabidopsis* mutants exhibiting altered production of carotenoids (*18*), flavonoids (*19*), tocopherols (*20*), and ascorbic acid (*21*) have been used to establish the genetic basis for their synthesis. The increasing ease of using expression in heterologous organisms has allowed functional cloning or characterization of steps in the synthesis of several vitamins and phytochemicals. The most successful has been the carotenoid pathway in which most of the biosynthetic enzymes have been cloned by color complementation in *Escherichia coli* (*22*). More recently, plant enzymes involved in iron uptake and biotin, thiamin, and vitamin E synthesis have also been cloned or functionally characterized with heterologous expression systems (*23*, *24*).

Utility of Genomics in Dissecting Plant Pathways of Nutritional Importance

The large number of ongoing sequencing projects in a variety of organisms represents one of the most significant developments for researchers in plant metabolism during the past two decades. Analysis of the growing DNA database shows a significant degree of interkingdom homology at the level of primary protein sequence (*25*). That many of these interkingdom orthologs are involved in basic cellular functions (for example, protein synthesis, cell division, primary carbon metabolism, and signal transduction) attests to the evolutionary conservation of these processes. Of the remaining sequences in an organism, about half have orthologs of unknown function in other organisms, while the remainder encode novel (pioneer) sequences with no related sequences in the database (*25*). Many unknown and pioneer sequences are likely to have species-, family- or kingdom-specific functions that have evolved for purposes unique to the particular organism or group of organisms. Given the large number of secondary compounds unique to the plant kingdom, many unknown and pioneer plant sequences likely encode structural enzymes and regulatory components of plant secondary metabolism.

Plant researchers are beginning to use genomic resources and the power of DNA microarrays to study all areas of plant biology, including metabolism (*26*). These

Table 2. Selected phytochemical classes, health-promoting properties, example active compounds, and good plant sources.

Phytochemical Class (no. of compounds)	Diseases ameliorated or prevented	Example active compound and plant source
Carotenoids (>700)	Prostate, esophageal and other cancers, cardiovascular disease, macular degeneration (*14*)	 Lycopene (tomatoes)  Lutein (kale, spinach)
Glucosinolates (>100)	Cancers (*12*)	 Glucoraphanin (broccoli and broccoli sprouts)
Phytoestrogens (>200)	Cardiovascular disease, osteoporosis, breast, prostate and colon cancers (*13*)	 Genistein (R=OH); Daidzein (R=H) (soybeans, tofu, soy products)
Phenolics (>4,000)	Cardiovascular disease, cancers (*42*)	 Resveratrol (red wine, red grapes)

technologies complement and can be readily integrated with existing biochemical and genetic approaches to add new dimensions to the elucidation of complex metabolic pathways in plants. The remainder of this review focuses on the progress and potential for using genomic technologies to aid in the identification of genes for plant secondary pathways of human nutritional importance. Because of the differing size and biosynthetic complexity of the two target compound classes (30 essential vitamins and minerals versus thousands of health-promoting phytochemicals), slightly different experimental approaches are discussed for each.

Nutritional Genomics: An Approach for Dissecting and Manipulating Micronutrient Pathways in Plants

Nutritional genomics is a general approach to gene discovery that is currently most applicable to compounds of nutritional importance that are synthesized or accumulated by plants and other organisms (for example, vitamins and minerals). Nutritional genomics takes advantage of the concept of metabolic unity among organisms through evolution. By using databases, protein and DNA homologies, and in silico computer searches it is possible to rapidly move experimentally between organisms while remaining focused on the single pathway or enzymatic reaction of interest from the target organism. The approach is broadly applicable and allows one to take advantage of a variety of model systems having specific attributes that may be lacking or underdeveloped in the target organism (for example, fully sequenced genomes, operons, pathway mutations, targeted gene disruptions, or functional complementation).

Identifying the genes needed to increase the levels of essential micronutrients in staple crops is an immediate goal that would have a significant impact on human nutrition worldwide. For example, because all plants synthesize vitamins, genes for their synthesis can be isolated and transferred from any plant system, including those being developed as genomic resources. Furthermore, these same vitamins are also produced in nonplant systems, such as bacteria and yeast, many of whose genomes have been fully sequenced. As such, previous biochemical, genetic, and molecular data for vitamin synthesis in nonplant systems can be readily accessed by genomics to identify putative biosynthetic orthologs in plant databases. Once identified, plant orthologs can rapidly be functionally tested by expression in the well-characterized auxotrophic bacterial mutants originally generated to elucidate the target vitamin pathway in these organisms.

The nutritional genomics approach has recently been applied to the vitamin E biosynthetic pathway in plants. The first step of the pathway was isolated from *Arabidopsis* with fungal and human orthologs as database queries (*23*). This *Arabidopsis* sequence provided a genomic stepping stone to identify an ortholog in a 10-gene operon in the photosynthetic bacterium *Synechocystis* PCC6803 (*27*). Gene disruption experiments showed that this operon also encoded the final step in vitamin E synthesis, γ-tocopherol methyltransferase (γ-TMT). The *Synechocystis* γ-TMT gene allowed isolation of an ortholog from the *Arabidopsis* database whose overexpression increased vitamin E levels nearly ninefold in *Arabidopsis* seed oil (*28*). This work demonstrates the power of applying genomics to dissect vitamin biosynthesis in plants and one strategy for modifying plant vitamin content. Genomics will help accelerate isolation of biosynthetic genes for most other plant vitamin pathways in the coming years. The use of these genes to manipulate plant nutritional content heralds an exciting new era for plant biochemists.

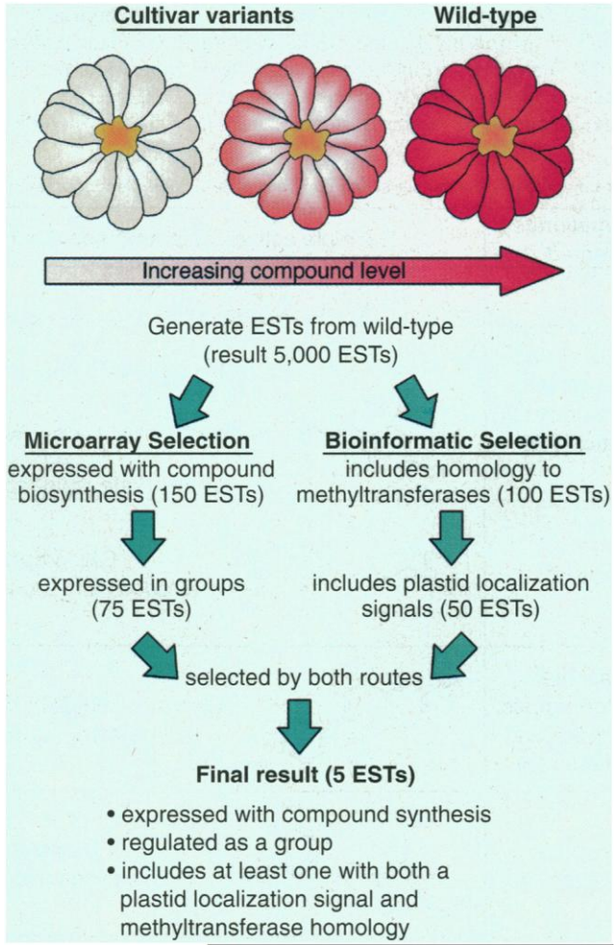


Fig. 1. The use of "nonmodel" plant systems to isolate candidate genes in phytochemical pathways. The hypothetical example illustrates isolation of candidate cDNAs for a chloroplast-targeted methyltransferase that is one of several steps involved in the synthesis of an orange-colored phytochemical found specifically in flowers. Wild-type flowers synthesize and accumulate the compound to high levels, 100-fold greater than cultivar variants. A nonredundant EST population is generated from wild-type petals during the highest rate of compound synthesis. Two subpools of ESTs are selected. Microarray selection uses RNA from roots and leaves (which do not synthesize the compound) and wild-type and cultivar variant flowers at identical developmental stages. ESTs coordinately regulated with compound synthesis and expressed in groups are selected. Bioinformatic selection identifies candidate ESTs by the presence of conserved methyltransferase motifs and plastid-targeting signals in the primary amino acid sequence. ESTs occurring in both groups are prime candidates for additional functional analysis.

Combining Bioinformatics and Microarrays to Elucidate Phytochemical Pathways of Nutritional Importance

Although nutritional genomics is a powerful approach for the limited number of plant compounds that are also synthesized in other model organisms (for example, vitamins), applying this approach to phytochemical pathways presents problems and limitations not encountered for vitamins. The extreme biochemical diversity and limited evolutionary distribution of many phytochemicals makes gene identification much more difficult. Orthologs for most phytochemical biosynthetic genes will be unique to the plant kingdom, which limits the usefulness of nonplant databases. Similarly, plants being developed as genomic resources (for example, *Arabidopsis*, maize, and rice) do not synthesize many of the best-characterized health-promoting phytochemicals and will therefore lack many target genes. For these and other reasons, researchers studying a specific phytochemical are often limited to a few plant species that synthesize and accumulate the target compound or compounds to high levels (for example, 0.5 to 5% of dry weight). Although biochemical flux through the

target pathway (and presumably the enzymes and their associated mRNAs) is elevated in such plants, they often lack genetic, genomic, and molecular tools found in model plant systems, which can severely hinder research progress. However, these plants do provide an information-rich resource of evolutionarily established, genome-wide expression patterns for the elevated synthesis and accumulation of a particular phytochemical. The question becomes: How can researchers access and use this information resource?

One possible approach, not yet fully implemented, applies the technologies of large-scale DNA sequencing and DNA microarray expression studies to nonmodel plant species and their natural variants that accumulate high amounts of a target compound (Fig. 1). By this approach, essentially any plant system or tissue can be used to generate an expressed sequence tag (EST) population that can be subjected to combined bioinformatic- and expression-based analyses to identify a limited set of candidate genes for a pathway. Several reports have demonstrated the power of microarray technology for uncovering coordinated gene expression patterns (*29*) and indicate that expression of unidentified genes in a pathway is often highly correlated with that of known pathway genes.

For gene identification by bioinformatics, the existing biochemical knowledge for individual pathway steps, well-characterized protein motifs common to specific reaction mechanisms, and the presence or absence of subcellular targeting information in the primary amino acid sequence are combined to select a subpool of candidates from the bulk EST population. For expression-based gene identification, DNA microarrays are used to analyze gene expression patterns in response to a large set of cultivar-, development-, tissue-, and stimuli-specific variations in target compound accumulation. Where some pathway genes are already cloned, these can act as pathway-specific markers for expression studies. Cluster analysis is then used to select an EST subpool whose expression is coordinately regulated with synthesis of the target compound. ESTs identified independently in both subpools would be prime candidates for enzymes of the target pathway. ESTs showing only closely coordinated regulation with target compound levels could be sequenced further to obtain additional useful information from primary amino acid sequence.

This general approach will facilitate the identification of a limited number of candidate genes pertinent to the pathway of interest for further study. Once identified, such genes (and orthologs) can be functionally analyzed by heterologous expression or knockout approaches in a variety of organisms including bacteria, yeast, maize, rice, and *Arabidopsis*. In addition, important nonpathway genes (such as those of known identity in primary and intermediary metabolism) whose expression is modified to enable increased flux or accumulation of the compound of interest will also be identified. In this way, rather than being a limitation, the unique biochemistry, flux, cell biology, and metabolic diversity present in "nonmodel" plant species can be used as a powerful resource to aid in dissecting a given pathway.

Concluding Thoughts

Research to improve the nutritional quality of plants has historically been limited by a lack of basic knowledge of plant metabolism and the often daunting task of dissecting whole branches of plant secondary metabolism. The advent of genomics provides new integrative approaches to plant biochemistry that allow crossing of species, family, and phyla barriers. As a result, the increase in our basic knowledge of plant secondary metabolism during the coming decade will be truly unparalleled and will place plant researchers in the position of being able to modify the nutritional content of major crops to improve aspects of human health. For essential minerals and vitamins that are limiting in world diets, the need and way forward is clear, and improvement strategies should be pursued, as long as attention is paid to the upper safe limit of intake for each nutrient (Table 1). However, for many other health-promoting phytochemicals, decisions will need to be made regarding the precise compound or compounds to target and which crops to modify such that the greatest nutritional impact and health benefit is achieved. Because these decisions will require an understanding of plant biochemistry, human physiology, and food chemistry, strong interdisciplinary collaborations will be needed among plant scientists, human nutritionists, and food scientists in order to ensure a safe and healthful food supply for the coming century.

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