

Agricultural Approaches to Improving Phytonutrient Content in Plants: An Overview

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Recent advances in plant molecular biology, functional genomics, and biochemistry have opened up a number of new avenues of research that will enable plant biologists to characterize, increase and modify plant content of a wide range of essential minerals and vitamins, as well as a number of secondary plant compounds that appear to play a role in improving human health and nutrition. In this review, several examples of exciting new research applying plant genomic and molecular genetic approaches to the improvement of phytonutrient content and composition in plants are presented. Research focusing on the elucidation of many of these complex biosynthetic and transport pathways in plants will require considerable resources in terms of funding, time, and personnel. As plant biologists move into interdisciplinary collaborations with nutritionists and food scientists, attention must be paid to a more complete identification and characterization of specific bioactive phytonutrients. Also, a more detailed assessment of the health-promoting properties of these compounds is needed, particularly for many of the secondary plant compounds for which clear epidemiologic and clinical data are still lacking. Finally, in order for significant progress to be made in modifying the nutrient composition of crops, a major investment must be made by funding agencies.

Introduction

There is considerable current interest in the role that plants and more specifically, plant-based foods, play in human nutrition and health. Recent shifts in dietary guidelines

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indicate that the typical American diet should contain a larger proportion of plant-based foods, i.e. vegetables, fruits, nuts, legumes, and grains.¹ While the preponderance of attention is currently focused on the secondary products in plant foods, attention must be focused as well on proteins; lipids; carbohydrates; and vitamins and minerals, particularly iron, zinc, and calcium, nutrients in which certain population groups in both the United States and worldwide are commonly deficient.^{1,2}

Plants synthesize and accumulate an astonishingly diverse array of organic secondary products, and data are beginning to accumulate indicating that a number of these organic compounds have health-promoting properties. Much of the current interest in phytonutrients has focused on these secondary plant products. At this time, little is known about the biosynthesis and accumulation of many of these compounds in plant tissues, and the specific bioactive forms within broad categories of secondary compounds have yet to be identified. However, recent technologic advances in plant genomics and biotechnology as well as the focused application of traditional and molecular-assisted plant breeding practices now provide plant biologists with an array of powerful tools to elucidate the mechanisms and regulation of the complex biosynthetic pathways for many of these compounds, and to begin to design strategies to alter the amount, distribution, and forms of those phytochemicals with health-promoting properties.

What Do We Mean by the Term Phytonutrient?

A literal definition for the term phytonutrient is "a nutrient derived from plants." Thus, we would be talking about a plant-based substance essential for proper metabolism and function in humans. This would include proteins, lipids, and carbohydrates as well as essential minerals and vitamins. However, the term is generally used much more loosely to include any organic or inorganic compound in plant foods that has a positive impact on human health or nutrition. This would include the true essential nutrients and vitamins such as vitamin E, pro-vitamin A, carotenoids,

iron, zinc, calcium, and selenium for which there is solid evidence as to their importance in human health and nutrition from research in animal model systems as well as from clinical and epidemiologic studies.^{3,4} Also included under this umbrella term would be the broad classes of secondary compounds such as phytoestrogens, isoflavonoids, anthocyanins, polyphenols, and glucosinolates that can improve human nutrition and health by mechanisms that are not, or are only partially, defined. A better term for these secondary plant products might be "accessory health factors" (GF Combs, personal communication). This term indicates that although not essential for humans, these compounds could play an important role in improving human health by reducing the impact of certain chronic diseases (e.g., heart disease, cancer) and the effects of aging.

Phytochemicals Within Plant Systems

The essential and non-essential phytonutrients are acquired, synthesized, translocated, and stored in specific plant tissues by a variety of processes and pathways. The essential minerals enter the plant primarily by absorption from the soil solution into plant roots. Mineral nutrients such as potassium, magnesium, calcium, iron, zinc, manganese, copper, and nickel are absorbed as metal ions, whereas nitrogen (N), phosphorus, and sulfur (S) are absorbed as oxyanions that are subsequently modified by reductive assimilatory pathways (for N and S) and incorporated into proteins, lipids and carbohydrates.^{5,6} Vitamins such as E, C, folate, and the pro-vitamin A carotenoids—as well as numerous secondary plant compounds—are synthesized via a number of different primary and secondary biochemical pathways. There are approximately 100,000 secondary compounds synthesized by different plant species that do not appear to play a direct role in plant growth and development.⁷ These compounds play roles in a diverse range of processes, including defense against herbivore and pathogen attack, protection against abiotic environmental stresses such as UV damage, and as attractants for pollinators.⁸ There are four general categories of secondary compounds:

- Phenolics, which are aromatic compounds that include the flavonoids, the largest group of phenolics. The flavonoids, which are synthesized via the shikimic acid pathway, can be sub-classified into different groups that include potentially important phytonutrients including isoflavonoids, flavones, flavonols, anthocyanins, tannins, and lignin.
- Terpenes, which include the carotenoids, steroids, and limonoids, are lipids that are made via the mevalonic acid pathway.
- Alkaloids, a diverse group of N-containing secondary compounds biosynthesized primarily from amino acids which include toxic and psychoactive plant compounds

such as nicotine, caffeine, cocaine, morphine, strychnine, and atropine.

- Non-alkaloid nitrogenous compounds, which include the glucosinolates and glycosides. The biosynthetic pathways for these different secondary compounds are usually quite complex, with numerous steps, branch points, and many closely related compounds in any particular group of compounds.

Experimental Framework for Engineering Plant Foods to Improve Human Health

Plant researchers moving into this field must be highly selective in choosing which plant compounds they manipulate, because of both the complexities of plant biology and the obvious demand for careful and methodologic alterations of plant nutrition value. However, at present, the roles and benefits for many of the secondary compounds of interest in plants are still poorly defined. In many of the clinical and epidemiologic studies, particularly for organic phytonutrients, a broad category representing a mixture of many related plant-based compounds has been shown to have potential for improving human health. Moreover, the specific bioactive species has often not been identified. Additionally, some of the relevant biosynthetic pathways have not been fully characterized at the biochemical and molecular levels, which limits the immediate possibilities for altering levels of intermediates of these pathways. Finally, many secondary compounds are found in specific plant species or plant families, some of which are not readily amenable to manipulation by molecular approaches.

An example of successful cooperation between plant biologists and human nutritionists comes from the exciting recent findings from the Jean Mayer USDA-ARS Human Nutrition Research Center on Aging at Tufts University. Researchers there have developed an assay to rapidly quantify antioxidant activity in different foods, and have found that highly colored fruits such as blueberries and strawberries had the highest antioxidant activity of any plant foods.⁹ Subsequent work strongly suggests that the antioxidant compounds in these fruits are anthocyanins, which are pigmented flavonoids belonging to the largest class of phenolic compounds in plants. Very recently, these same researchers showed that in long-term feeding studies of rats, supplementation of diets with extracts from antioxidant-rich fruits and vegetables appeared to retard age-associated declines in the central nervous systems as well as cognitive behavioral deficits.¹⁰ These findings clearly point out a potentially productive and important avenue of research for plant biologists. However, the specific antioxidant compound has not yet been identified, and it could be one or more members of a large number of anthocyanins produced by a pathway that is presently not clearly delineated. Thus, rapid progress

terms of increasing the content of specific anthocyanins in order to enhance the anti-aging properties of a particular plant food is not immediately forthcoming, simply because plant biologists cannot yet target a specific compound.

In contrast to the difficulties encountered by plant biologists in modifying anthocyanins in fruits, a successful genomics-based approach was recently used to metabolically engineer the vitamin E (α -tocopherol) biosynthetic pathway in plant seeds.¹¹ This was considered meaningful to human health because of the tendency for many Americans to consume less vitamin E than is recommended. These research findings hold great promise for increasing the vitamin E content of agriculturally important oil crops and are a good example of the potential that exists for manipulating phytonutrients in plants.

When research priorities are developed for determining which phytonutrients should be targeted, the essential minerals and vitamins listed above should be given careful consideration. For example, only 21% of the diets of teenage girls in the U.S. meet the Recommended Dietary Allowances (RDAs) for iron, 16% meet the RDAs for calcium, and many do not meet RDAs for magnesium, zinc, vitamin A and vitamin E.¹ Iron is an excellent example of an essential phytonutrient that could have a great positive impact on human health if more iron was available from plant foods. There are significant segments of the U.S. population that are iron deficient. Up to 20% of all premenopausal women in the U.S. have low hemoglobin levels, and this increases to 40% for pregnant, low-income African-American women.¹ This has important implications for the United States, as recent research indicates that iron deficiency during pregnancy may retard the cognitive development in the young child of an iron-deficient mother. This problem is even more severe in developing countries as iron deficiency is the most prevalent mineral nutrient deficiency in the world, affecting more than 2 billion people worldwide.² The global extent and severity of this problem indicates that research aimed at enhancing the iron fortification of staple foods such as cereal grains should have a high priority both for developing nations and for developed countries such as the United States.

It is clear that if phytonutrient research is to develop and succeed, better interdisciplinary linkages are needed between experts in plant biology, human nutrition, and food science. Plant biologists need to be continuously informed about new evidence on the biologic roles of plant foods in the human system. Similarly, nutritionists need to know more about the possibilities for biochemical and molecular manipulation of the phytonutrient content of plants, as well as about the obstacles. We can envision an iterative process in which nutritionists identify specific phytonutrients to be targeted by plant biologists, who

alter the content and/or form of the compound, and the modified plant food is then tested with animal or in vitro models, or in clinical trials. The findings from these trials would feed back to the plant biologists for additional modification in plants. Several ARS laboratories already exist that are models for such interdisciplinary links. For 60 years, the U.S. Plant, Soil and Nutrition Laboratory at Cornell University has brought together soils scientists, plant biologists and nutritionists to focus on a lab charter to investigate plant and soil factors that have an impact on human nutrition and health. More recently, the USDA-ARS Children's Human Nutrition Center in Houston has added several experts in plant biology to their laboratory staff of medical doctors, nutrition scientists, dietitians, and chemists to investigate problems in maternal, infant, and child health and nutrition.

Agricultural Approaches for Improving Phytonutrients in Plants

The current and ongoing revolution in biology regarding structural and functional genomics has provided plant biologists with a powerful new array of approaches and tools that should allow them not only to elucidate the complex transport and biosynthetic pathways for specific phytonutrients, but also to modify the amount, form and location of these compounds in specific plant tissues and organs.^{12,13} Additionally, the focusing of plant breeding approaches on the identification and exploitation of genetic variation for phytonutrient-related traits is already yielding progress.

Plant Breeding Approaches

As plant breeders and plant molecular geneticists begin to direct research toward phytonutrients in crop plants, they are finding significant genetic variation in the content of specific phytonutrients. This variation is currently being exploited to enhance phytonutrient levels through both traditional and marker-assisted plant breeding.¹⁴⁻¹⁶ A good example of this is the breeding effort that has yielded carrot germplasm with increased levels of provitamin A carotenoids.^{16,17} This approach allows plant breeders to implement phytonutrient modification (for those compounds for which significant variation exists) and is covered in more detail in several of the other reviews in this publication.^{18,19}

Another approach used by plant breeders is the use of induced mutagenesis to create new variability for a trait when existing natural variation is not adequate. Because of its inherent inefficiency, mutation breeding has historically played a minor role in crop improvement. However, now that we are armed with a sophisticated understanding of plant biochemistry and with methods and protocols for rapid analysis of chemical composition, it is sensible to revisit the use of induced mutagenesis as an approach

to altering phytonutrient composition in crops. One major factor that will aid in the successful implementation of mutagenesis to favorably alter phytonutrient composition is that in many instances, target phytonutrients may be non-essential to plant productivity because they are products of secondary metabolism. As such, it should be possible to induce dramatic shifts in their abundance without detrimentally affecting crop performance or quality.

One group of compounds for which mutation breeding may find an application is the flavonoids. Following the first committed step of flavonoid synthesis (the condensation of malonyl-CoA and coumaryl-CoA to form chalcone), the different classes of flavonoids—including anthocyanins, flavones, and proanthocyanidins—are synthesized. As discussed previously, it has been suggested that some of these flavonoids have health-promoting properties. In contrast, other flavonoids have been found to have a deleterious impact on nutrition, at least in animals. For example, high proanthocyanidin content is associated with poor feed quality in sorghum.

A useful example of how mutagenesis can be used to favorably alter flavonoid composition comes from research conducted more than 20 years ago at the Carlsberg Laboratories in Denmark. Mutagenesis with compounds such as EMS has been used to identify barley mutants with altered flavonoid composition. These mutants represent at least 29 complementation groups (and thus, 29 distinct loci).²⁰ From a basic standpoint, the mutants have been invaluable in deciphering the biosynthetic interrelationships of the different flavonoids in barley. This has been a difficult issue to address because some steps in flavonoid biosynthesis are reversible and because certain flavonoids may be synthesized from more than one point in the pathway. Biochemical analyses of the different mutants made it possible to determine the different steps in flavonoid biosynthesis that are genetically controlled, and the sequence in which they occur.²⁰ This was accomplished by examining the flavonoid composition of the different mutants for the preferential accumulation of certain compounds and the loss of others.

There is also practical significance to some of the barley mutants that have an altered flavonoid composition. First, those mutations that specifically eliminate proanthocyanidin accumulation are of great interest to the beer brewing industry. Barley proanthocyanidins strongly react with proteins in the beer and form a precipitate that has to be removed by chemical methods. Brewing beer with grain from barley mutants lacking proanthocyanidins sidesteps the formation of these precipitates, thus providing a simple means of eliminating certain chemical steps from the brewing process.²¹ Second, one of the classes of barley flavonoid mutants lacks dihydroflavonol reductase activity, and thus accumulates the substrate for this enzyme, dihydroquercetin. Height-

ened accumulation of this compound has been found to inhibit infection by certain pathogenic fungi.²² While these two examples do not directly relate to human health, they nonetheless demonstrate that the spectrum of flavonoids in a plant can be specifically altered by mutagenesis breeding. As such, it should be possible to apply this approach in crops to enhance levels of flavonoids or other compounds that act as phytonutrients for humans, or to eliminate compounds deleterious to human health.

Bioengineering of Selected Biosynthetic Pathways

The tools becoming available from plant genomics and for tissue specific gene transformation of many plant species provide new avenues for plant biologists to dissect out the complex biosynthetic pathways for the synthesis of many phytonutrients. They can also use these tools to selectively modify specific intermediates in these pathways. A good example of this is the recent success by Shintani and DellaPenna in using a genomics-based approach to clone a key enzyme in the synthesis of vitamin E in plants, and then to overexpress this enzyme in seeds in order to elevate seed vitamin E content.¹¹ Vitamin E, or α -tocopherol, is a lipid-soluble phenolic compound synthesized only in higher plants and some photosynthetic bacteria. There are four tocopherols made in plants— α -, β -, γ -, and δ -tocopherol—with α -tocopherol having the highest vitamin E activity and thus being the most important for human health. Much of the vitamin E in the human diet comes from vegetable oils, derived from the seeds of soybean, maize, cottonseed, and the rape plant. In all of the major oilseed crops, α -tocopherol is found in relatively low levels, while its immediate biosynthetic precursor, γ -tocopherol is found at much higher concentrations (10- to 20-fold higher). These findings suggest that the final step in α -tocopherol synthesis, which is catalyzed by γ -tocopherol methyltransferase (γ -TMT), is limiting in the seeds of the major oil crops.

The α -tocopherol biosynthetic pathway has been described and one of the enzymes of the pathway, p-hydroxyphenylpyruvate dioxygenase (HPPDase), has recently been cloned from *Arabidopsis* and carrot.^{23,24} However, it has been difficult to clone the genes encoding the other pathway enzymes, as they are all membrane-bound. Therefore, these researchers used a genomics-based approach to clone the γ -TMT gene by using sequence similarities between *Arabidopsis* and the photosynthetic bacteria *Synechocystis* PCC6803, for which the entire genome has been recently sequenced. The gene encoding the *Arabidopsis* HPPDase was used to search the *synechocystis* genome database, and in this way the homologous *synechocystis* HPPDase gene was cloned and found to lie within a predicted 10 gene operon. As bacterial biosynthetic genes are often organized in operons, the authors

speculated that the gene encoding γ -TMT might be nearby to the cloned BPPDase gene. A candidate gene that predicted a protein with high amino acid sequence similarity to plant methyl transferases was found nearby and cloned. A null mutant for this gene was created, and biochemical analysis of tocopherol synthesis in the mutant as well as enzyme activity studies with the recombinant protein showed that indeed, the gene encoded γ -tocopherol methyltransferase.

This allowed the researchers to use the synechocystis γ -TMT protein sequence to search the Arabidopsis expressed sequence tag (EST) data base, which enabled them to clone the homologous gene from Arabidopsis. Subsequently, the Arabidopsis γ -TMT was overexpressed in transgenic Arabidopsis plants behind a seed specific promoter and the resulting plants showed a dramatic shift in their tocopherol accumulation. In seeds of wildtype Arabidopsis, as in the oilseed crops, >95% of the total tocopherol exists as γ -tocopherol. In the transgenic plants, there was a greater than 80-fold increase in seed α -tocopherol content, and >95% of the total tocopherol was α -tocopherol. If, as expected, γ -TMT activity in the commercially important oilseed crops is also limiting, this approach should significantly increase seed α -tocopherol levels and will most likely enhance the nutrition value of these crops as sources of vitamin E. This is an elegant example of the potential that exists to modify phytonutrient content and composition in plant foods.

Expression of Phytonutrient Genes in Plants

It may be possible to use plant biotechnology methodologies to enhance phytonutrient content in specific plant tissues without having a complete or detailed understanding of the complex transport or biosynthetic pathways for the phytonutrients. For example, there is considerable interest in elevating the levels of iron in staple foods such as the seeds of rice and maize, in order to begin to address the widespread iron deficiency in developing countries.² Normally, plant seeds are poor sources of iron. The pathway for iron from the soil to the developing seed is quite complex and poorly understood, involving a number of cell types and both membrane and long-distance transport systems. Genes encoding the plant iron storage protein (phytoferritin) have been cloned,²⁵ and a number of labs are actively pursuing the overexpression of the phytoferritin genes in seeds of cereal crops with the hope of providing a biotechnological solution to iron fortification of staple plant foods.

One recent example of this biotechnology approach, which holds promise for increasing the pro-vitamin A carotenoid content of staple foods such as rice, comes from the recent work of Burkhardt and colleagues.²⁶ Rice endosperm contains neither β -carotene nor any of the immediate precursors for the biosynthesis of β -carotene. In this study, however, biochemical analysis of immature rice

endosperm revealed the presence of geranyl diphosphate, which is a 20-carbon isoprenoid precursor necessary for carotenoid synthesis. Therefore, in an effort to improve the nutrient quality of rice, the gene encoding the enzyme phytoene synthase from daffodil was expressed in rice using an endosperm specific promoter. Phytoene synthase is the first of 4 specific enzymes needed for the synthesis of β -carotene in plants. The researchers were successful in expressing the active phytoene synthase enzyme and in synthesizing phytoene in rice endosperm. This research demonstrated that it is possible to engineer a critical step for carotenoid synthesis in a non-photosynthetic tissue that normally is devoid of carotenoids. These findings have opened the way for transforming rice endosperm with the remaining three enzymes needed for β -carotene synthesis, which may have a large impact in lessening vitamin A deficiency in developing countries that depend on rice as a staple food.

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