ISB NEWS REPORT
AGRICULTURAL AND ENVIRONMENTAL BIOTECHNOLOGY

March 2015

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PLANT RESEARCH

Development of Biofortified Maize through Molecular Breeding

HS Gupta, Firoz Hossain, and V Muthusamy

Micronutrient malnutrition, commonly known as 'hidden hunger' mainly due to iron, zinc, and vitamin A deficiencies, has emerged as one of the major health problems worldwide[1]. Of these micronutrients, vitamin A deficiency (VAD) results in visual impairment and higher morbidity as well as mortality in at least 190 million pre-school children and 19 million pregnant women, mostly in Africa and South Asia. Although many strategies including supplementation, dietary diversification, and commercial fortification of foods have been deployed to overcome VAD, biofortification involving crop varieties that are rich in micronutrients promises to be a cost-effective and sustainable approach, which provides consumers the essential micronutrients in their natural form[1]. Maize is one of the most important staple foods consumed by more than a billion people in sub-Saharan Africa, Latin America, and Asia. By 2050 the demand for maize in the developing world is likely to double. Considering the growing importance of maize as both food and feed, biofortification of maize through enhancement of provitamin A, coupled with superior protein quality, assumes worldwide significance. The present article describes development of β-carotene-rich quality protein maize (QPM) hybrids through molecular breeding.

Quality Protein Maize (QPM)

Maize protein is deficient in two essential amino acids, lysine and tryptophan. Humans and monogastric animals such as poultry birds cannot synthesize these amino acids in their body; they are therefore required in the diet. Identification of the nutritional benefit of recessive *opaque2* mutant present on chromosome 7, followed by accumulation of endosperm-modifiers, led to development of QPM at the International Maize and Wheat Improvement Center (CIMMYT), Mexico[2]. The biological value of QPM is 80% as compared to 45% of normal maize; and the protein quality of QPM is 90% of that of milk.

Several QPM hybrids have been developed at CIMMYT and introduced in the developing world. However, development of new QPM hybrids with indigenous germplasm requires conversion of normal maize inbred lines to QPM inbreds through transfer of *opaque2* allele. Transfer of recessive alleles in otherwise agronomically superior genotype through conventional breeding requires progeny testing after each generation of backcrosses. However, utilization of molecular markers associated with the target allele helps in selecting the desirable segregants in each backcross generation, thereby considerably shortening the breeding cycle. Further, deployment of genome-based markers assures high recovery of the recurrent parent genome (RPG) within just two backcross generations.

Availability of gene-based SSR markers for *opaque2* facilitates the accelerated development of QPM through marker-assisted backcross breeding strategy. We undertook the conversion of

Vivek Hybrid-9 (an early maturing high yielding commercial maize hybrid) to QPM using marker-assisted selection (MAS) at ICAR-*Vivekand* Institute of Hill Agriculture in India. The parental inbreds CM212 and CM145 were improved (by introgression of *opaque2* allele) to VQL1 and VQL2, respectively[3]. These inbreds possess high RPG achieved through stringent background selection. The reconstituted version of Vivek Hybrid-9, named Vivek QPM-9, possesses 30% greater lysine and 41% more tryptophan while retaining the same level of productivity of the original hybrid. Vivek QPM-9 released for commercial cultivation earns the distinction of being the first MAS-based maize cultivar released in India. Currently, Vivek QPM-9 is one of the promising QPM hybrids with adaptability to both hilly and plain regions.

Breeding for higher kernel β-carotene

White kernel maize lacks carotenoids due to the presence of recessive *y1* (non-functional *phytoene synthase1*) in homozygous state, whereas yellow maize produces enough carotenoids due to conversion of the first committed step in carotenoid biosynthesis pathway by dominant *Y1* – an active *phytoene sysnthase1*. In addition, yellow kernel maize exhibits tremendous natural variation for both provitamin A (α-carotene, β-carotene and β-cryptoxanthin) and non-provitamin A (lutein and zeaxanthin) carotenoids, and holds promise for biofortification of provitamin A.

Based on factors such as a bioavailability ratio of 12:1, retention up to 50% after processing, nutrient status of the host, food matrix, and amount of food consumed in the meal, nutritionists have fixed a goal of 15 µg of provitamin A per unit of dry weight of maize kernel in the biofortification program (www.harvestplus.org). Multilocation evaluation of a diverse set of 115 maize inbreds from Indian- and CIMMYT-breeding programs reveals the existence of a wide variation of carotenoids amenable to genetic improvement through breeding. Our experiments[4] show that the traditional maize inbreds are low in β-carotene (mean: 0.5 µg/g; range: 0.0 to 1.8 µg/g) coupled with a smaller proportion of β-carotene (3%) to total carotenoids, as compared to zeaxanthin (53%), lutein (35%) and β-cryptoxanthin (9%). In contrast, the newly developed CIMMYT-HarvestPlus inbreds show much higher β-carotene (mean: 12.1 µg/g; range: 8.9 to 15.0 µg/g), indicating their potential as donors in the β-carotene enrichment program. However, the challenge for enrichment of β-carotene in maize lies in large-scale phenotyping for kernel carotenoids, as high performance liquid chromatography (HPLC) is expensive. Moreover, kernel color is not a reliable indicator of β-carotene concentration, thereby limiting its use in indirect selection[5]. Therefore, a MAS for desirable allele(s) that enhance accumulation of β-carotene in kernels can be of immense use.

Carotenoid biosynthesis pathway

The carotenoid biosynthesis pathway is well characterized in maize. Among the genes involved in the pathway, *phytoene synthase1* (*Y1*) plays a pivotal role by condensing two geranylgeranyl pyrophosphate molecules into a single molecule of phytoene. The first branching point of the pathway is the cyclization of lycopene: the *lycopene epsilon cyclase* (*lcyE*) gene located on chromosome 8 converts more lycopene to the β, ε branch, which produces more α-carotene and lutein. The rare but naturally existing mutant allele of *lcyE* diverts more lycopene to the β, β branch that in turn produces

PUBLISHED BY

Information Systems for Biotechnology

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more β-carotene, β-cryptoxanthin, and zeaxanthin[6]. The large amount of β-carotene thus produced is further hydroxylated by *β-carotene hydroxylase* (*crtRB1*) to produce β-cryptoxanthin (having provitamin A activity only half that of β-carotene) and zeaxanthin (having no provitamin A activity). A recently discovered rare natural genetic variation in the *crtRB1* gene (present on chromosome 10) increases β-carotene by blocking hydroxylation step[7]. Large-scale validation experiments indicate that one favorable allele alone, namely *crtRB1* 3'TE, is responsible for causing 2- to 10-fold variation in β-carotene concentration in maize. Co-dominant markers for both *lcyE* and *crtRB1* genes have been identified using polymerase chain reaction (PCR) and would help to accelerate development of provitamin A-rich maize through MAS.

Variation for favorable *lcyE* and *crtRB1* alleles in Indian maize germplasm

Naturally occurring mutant alleles of *lcyE* and *crtRB1* associated with a higher accumulation of β-carotene (favorable allele) over other carotenoid components have been reported in exotic maize germplasm. We found a low frequency of the favorable alleles of *lcyE* (3.4%) and *crtRB1* (3.9%) while screening of a large set of Indian inbreds using gene-based markers. Interestingly, the inbreds had a lower concentration of β-carotene despite the presence of favorable alleles. Nucleotide polymorphism analyses in the 3'-UTR region of *crtRB1* identified two SNPs (*SNP4 and SNP13*) and two InDels (*InDel6 and InDel7*), which clearly differentiated high and low β-carotene genotypes possessing a favorable allele[8]. These nucleotide variations could be associated with the variation in kernel β-carotene accumulation by regulating translation and stability of mRNA.

crtRB1-based inbreds used in β-carotene enrichment program

Inbreds with a favorable allele of *crtRB1* have been used to develop hybrid combinations. Considering the genetic relationship, we crossed inbreds from India with inbreds of CIMMYT-HarvestPlus program. CIMMYT-HarvestPlus inbreds possessed a much higher concentration of β-carotene (11.3 µg/g) compared to the inbreds of Indian origin (1.6 µg/g). Hybrid combinations exhibited higher β-carotene (mean: 3.8 µg/g; maximum: 6.2 µg/g) compared to commercial hybrids used as check (mean: 1.8 µg/g); however, the β-carotene level in the experimental hybrids was much less as compared to CIMMYT-HarvestPlus inbreds used as parents. The favorable effects of the allele from CIMMYT-HarvestPlus origin could have been possibly masked/affected by the allele from the Indian origin. Though the hybrids generated were homozygous for the favorable allele of the *crtRB1* gene, the alleles from both parents differed in their nucleotide sequence[8], thereby possessing a heterozygous state for the allele. A favorable allele in homozygous condition is three-times more effective than in the heterozygous condition for accumulating kernel β-carotene[9]. Thus, the allele from CIMMYT-HarvestPlus origin must be in homozygous condition to gain the maximum benefit of the allele effect. Considering this, marker-aided introgression of this allele into elite maize inbreds would be an effective strategy for a provitamin A enrichment programme[10].

Marker-assisted selection for enrichment of kernel β-carotene in QPM

At the ICAR-Indian Agricultural Research Institute (IARI), New Delhi, Vivek QPM-9 (VQL1 × VQL2), the first QPM hybrid developed through MAS in India, was further targeted for the introgression of a favorable allele of the *crtRB1* gene. This hybrid possesses high grain yield potential with high tryptophan and lysine content but contains low levels of kernel β-carotene. Inbreds developed under CIMMYT-HarvestPlus program with the *crtRB1* 3'TE favorable allele and high kernel β-carotene served as the donors for introgression of the target allele. Foreground selection for the favorable allele of the *crtRB1* gene and the background selection for RPG recovery were carried out using polymorphic SSR markers. The backcross-derived progenies homozygous for the target allele and with high RPG were selected for phenotyping for kernel quality traits.

The concentration of β-carotene varied from 16.4 to 17.8 µg/g among the VQL1-based MAS-derived progenies, while it was 16.3 µg/g in the VQL2-based progenies (**Fig. 1**). A maximum 12.5-fold increase of kernel β-carotene was achieved upon introgression of the favorable allele of the *crtRB1* gene[10]. This higher increase of β-carotene in the recurrent parents upon introgression of the favorable allele indicates the major effect of the gene in enhancing the concentration of β-carotene[11]. These β-carotene enriched MAS-derived progenies also had similar lysine content to their recurrent parent. The average tryptophan in endosperm protein of VQL1- and VQL2-based progenies was 0.53 and 0.56 % respectively; similar to the recurrent parent VQL1 (0.54%.) and VQL2 (0.58%). In addition to a higher concentration of β-carotene and tryptophan, the MAS-derived inbreds also had a high degree of similarity to their recurrent parents for most of the morphological- and yield-attributing characters[12]. This is due to the high recovery of RPG achieved through genome-based SSR markers used in the background selection. However, a few marked differences were observed among the introgressed progenies, such as the absence of silk pigmentation in the VQL2-based progeny compared to the purple pigmentation in the recurrent parent VQL2. One of the VQL1-based introgressed progenies had higher cob placement than the recurrent inbred VQL1. These morphological characters will be useful in the registration process as they unambiguously differentiate the *crtRB1*-derived introgressed inbreds from the original inbreds during seed certification process.

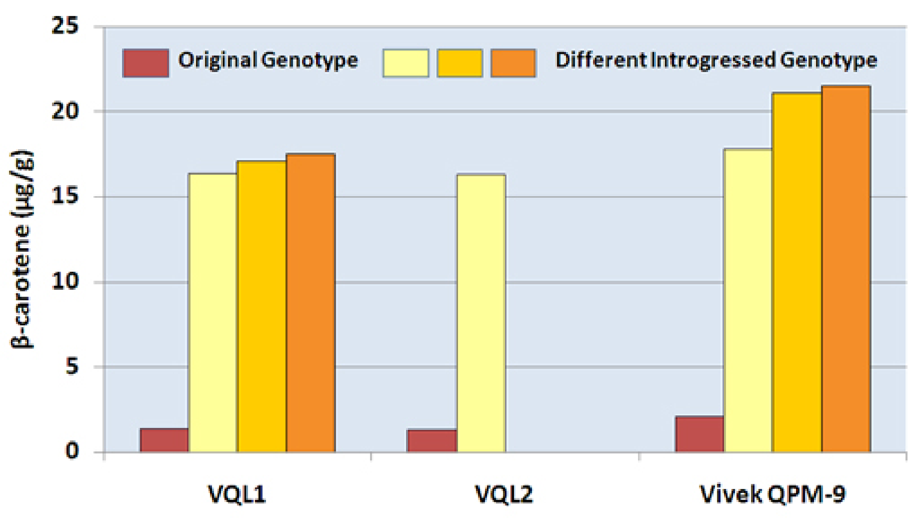

Figure 1. Kernel β-carotene in original and introgressed versions of Vivek QPM-9 and its parental inbreds

The MAS-derived inbreds with a high concentration of β-carotene and tryptophan and high morphological similarity were used to reconstitute the hybrids. The hybrids were tested at two different maize growing zones of India, viz. Delhi and Dharwad, and showed a significant increase in kernel β-carotene over their respective original hybrids. Three different versions of the reconstituted hybrids were evaluated, and kernel β-carotene ranged from 17.8 to 21.5 μg/g, compared to the original hybrid Vivek QPM-9 (2.1 μg/g)[10] (**Fig. 1**). An average of 9.6-fold enhancement of kernel β-carotene was observed among the introgressed progenies. These hybrids also recorded a similar concentration of tryptophan (0.81%) compared to the original hybrid (0.83%), as MAS for *opaque2* was also undertaken during the backcross breeding program. The hybrids also exhibited a similar grain yield potential and other yield-attributing characters compared to the original hybrid Vivek QPM-9 (**Fig. 2**). The β-carotene enriched MAS-derived hybrids recorded a grain yield of 5.6 to 6.0 tons/hectare at Delhi, and 5.7 to 6.1 tons/hectare at Dharwad, compared to the original hybrid Vivek QPM-9 (6.1 and 5.1 tons/hectare at Delhi and Dharwad, respectively)[10]. This hybrid is presently under testing in the All India Coordinated Maize Improvement Project.

Genetic improvement of Vivek Hybrid-9 to Vivek QPM-9 for protein quality, and its further conversion to provitamin A rich version of Vivek QPM-9, is a classic example of an incremental breeding strategy, where *opaque2* and *crtRB1* alleles were sequentially introgressed to develop the multi-nutrient rich maize hybrid. Development of the β-carotene-rich version of Vivek QPM-9 is the first successful demonstration of deployment of MAS for β-carotene enrichment in the QPM genetic background. Using MAS, three more commercial normal maize hybrids (Vivek Hybrid-27, HM-4, and HM-8) have also been biofortified for kernel β-carotene. Currently, breeders at ICAR-IARI, New Delhi, are involved in the introgression of favorable alleles for both *crtRB1* and *lcyE* into commercial QPM hybrids of medium and late maturity. Globally, five maize hybrids and three composites having 6 to 8 μg/g of provitamin A content have been released in Zambia, Nigeria, and Ghana. Development of the β-carotene-rich version of Vivek QPM-9 with a much higher level of β-carotene would be of immense value in alleviating VAD in India.

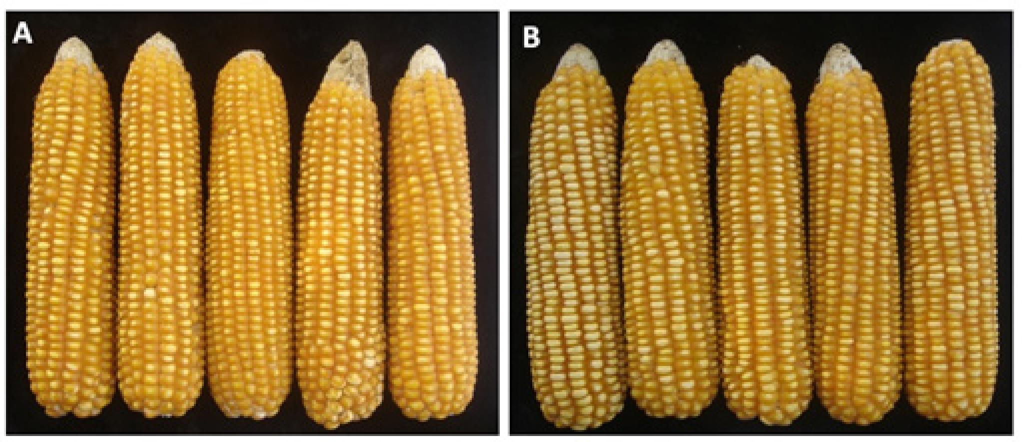

Figure 2. Cob characteristics of original (A) and β-carotene rich (B) versions of Vivek QPM-9

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Transgenic Soybean Seeds Accumulate β-Carotene

Monica Schmidt

The adage, 'you are what you eat' has long been part of consumer culture. Today with a heightened awareness of diet-related causes of cancer, obesity, and heart disease, consumers are increasingly interested in health-promoting foods. The phrase 'functional foods' has recently been added to the public vocabulary to describe food items that deliver health benefits beyond their basic nutrition. Foods boldly labelled as 'potent antioxidant', 'may reduce cholesterol', 'no trans fats', and 'high in omega 3' line grocery stores shelves.

Due to its prominence in global diets, soybean could have a significant worldwide impact as a functional food. Approximately 25% of the world's edible oils and 75% of the world's protein meal are derived from soybean. We have metabolically engineered soybean with enhanced levels of the nutraceutical carotenoid β-carotene accumulated in its seeds[1]. Interestingly, the β-carotene seeds also have an altered oil composition – an increase of oleic acid – as well as an elevated (4–8%) overall protein content. Hence our research achieved an improvement in three desired traits simultaneously in soybean seeds – elevated levels of β-carotene, oleic acid, and protein. Each trait addresses a consumer and/or processor need and will contribute to satisfying the growing global food demands.

Increased β-carotene accumulation in transgenic soybean

Carotenoids are potent antioxidants that reputedly help prevent aliments due to oxidative damage. Although there are over 700 carotenoids identified in nature[2], only the consumption of six of them, α-carotene, β-carotene, lutein, lycopene, zeaxanthin, and astaxanthin, have been shown to have health benefits. β-carotene might be the best known of the nutraceutical carotenoids. β-carotene is pro-vitamin A, which, after its consumption, is converted to its active vitamin A (retinol) form in the intestine.

Vitamin A deficiency is prevalent in developing countries where up to a half million children under the age of 5 years lose their sight, and half of those die within a year (www.who.int/nutrition/topics/vad/en/). In response to the need for more dietary vitamin A, 'Golden Rice'[3] was the first staple crop engineered to contain increased levels of β-carotene. Soybean seeds naturally contain β-carotene, a carotenoid that plays a pivotal role in harnessing light energy in photosynthesis, making soybean a good target candidate for β-carotene enrichment.

The rate-limiting step for the production of carotenoids in soybean is the conversion of geranylgeranyl diphosphate to phytoene, as this is the first committed step in the carotenoid pathway. Geranylgeranyl diphosphate is located at a significant branch point in a few metabolic pathways, and it is the substrate for a number of plant compounds such as chlorophyll, tocopherols, gibberellins, and carotenoids. To increase the flux from geranylgeranyl diphosphate down the carotenoid pathway, we engineered soybean to express a bacterial phytoene synthase gene with high catalytic activity targeted to the chloroplast in a seed-specific manner. The resultant genetically engineered soybean seeds contain up to 856 µg β-carotene/g (dry weight), which is an increase of 1,500-fold over wild-type levels.

For perspective, the level of β-carotene obtained in these soybean seeds is 15 fold greater than the levels obtained in the best Golden Rice[3] variety, twice the level measured in baby carrots, and to date, the highest level achieved in any transgenic system with a single transgene. Red palm oil, a major source of vitamin A in developing nations, contains total carotenoids of approximately 500–700ppm, almost half of this is β-carotene[4]. Our modified soybean seeds have a comparable level of β-carotene to red palm oil (845ppm average), and are thereby able to deliver the same vitamin content without the rainforest or orangutan habitat destruction associated with red palm oil harvests.

Other potential benefits of β-carotene enhanced soybean

Though an increased level of β-carotene, or pro-vitamin A, in soybean is useful in itself as a vitamin source, there are other benefits available from a β-carotene improved soybean. For instance, the high levels of β-carotene in soybeans could also be utilized as a metabolic feedstock bioreactor to produce additional carotenoids. For example, engineered β-carotene in soybean seeds could be used as a precursor for further modification of the isoprenoid pathway to produce other nutraceutical carotenoids such as zeaxanthin. The daily consumption of milligram amounts of both zeaxanthin and lutein have been noted to aid in the prevention of age-related macular degeneration, the leading cause of irreversible vision loss in elderly Americans[5].

Other potential applications of β-carotene as a feedstock could include engineering the accumulation of the carotenoid astaxanthin, a potent antioxidant with dietary applications and used as a component of shrimp and salmonoid feed, producing the signature red color. Much of the astaxanthin currently used as a feed supplement is synthetically produced, which is a significant input cost in salmon production. Astaxanthin inexpensively produced in soybeans could allow the broader use of this carotenoid as a food and feed antioxidant.

Healthier fatty acid profile

In addition to have a higher biologically relevant levels of β-carotene, our seeds had a higher level of oleic acid and a lower level of linoleic acid, which translates to lower trans fat content when the oil is processed. Coronary heart disease is a leading cause of death for Americans and has been estimated to cost the US nearly $109 billion/year, considering the health care costs, medications, and loss of productivity. Consumption of trans fats increases the risk of coronary heart disease by raising levels of LDL cholesterol and lowering the levels of HDL cholesterol. Hence, foods made with soybean oil containing this improved fatty acid composition might contribute to the reduction of the incidence of coronary heart disease. This reduction could be significant, as 25% of the calories consumed in the US are derived from plant oils[6].

Background on trans fats

The deleterious health aspects of trans fats are widely known to the point that there have been class action lawsuits filed against food manufacturers to reduce or eliminate trans fats from their products. In addition, mandatory food labeling for trans fats is required in several countries[7]. Technically a trans fat is an unsaturated fatty acid in a trans isomer configuration. They are largely produced in industrial food processing, such as baking or frying, that use polyunsaturated oils. Chemical hydrogenation revolutionized the oil industry decades ago, becoming largely popular because it produces a flexible mixture of fats that are solid at room temperature but liquid at the higher temperatures used for baking or frying. The commercial hydrogenation process is largely incomplete so some double bonds in the fatty acids re-form. In theory each double bond has an equal opportunity to re-form in either the cis or trans configuration, but due to thermodynamics, the trans isomer forms most of the time, as it is a lower energy state than the cis counterpart. Consequently, foods fried or baked with hydrogenated oil likely contain trans fats.

If soybean oil were modified to contain a lower compositional amount of unsaturated oils, hydrogenation for most applications would become unnecessary and the production of trans fats would be averted. Again, the soybean seeds we engineered to have higher levels of β-carotene also had an altered oil composition, with significantly reduced levels of unsaturated fatty acids. This effect is independent of the suppression modification approach currently deployed in commercial transgenic and breeding lines developed by industry, academia, and the USDA.

Benefits of protein enhancement

Oil and protein are major economic products from soybean seed. Nearly 85% of the global 251 million tons of soybean protein meal produced is used as animal feed (apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf). It is projected that the need for animal feed will increase over 235% by 2050. Our β-carotene-enhanced seeds contained an overall increase (4 - 8%) in protein content. Soybean seeds that contain increased protein content could help meet the protein demand for feeding feedstock in the future.

In addition to providing a greater quantity of protein, these engineered seeds may also afford the opportunity to produce more functional proteins. Functional proteins produced in soybean could include vaccines intended for direct consumption by consumers. In addition, the engineered soybeans could be used as biofactories to make a vaccine protein effectively and inexpensively. Other proteins targeted for increased production in soybean feed could include industrial feed enzymes, such as phytase used to break down indigestible phosphates.

There is a well-documented negative correlation between oil and protein concentrations in soybean seeds. The biological reason for the negative protein/oil correlation remains unknown and is a focus of further research that may lead to improvements in seed composition. This discovery of a connection between lipid soluble carotenoid production and oil and protein content contribute to the knowledge base in this area of basic research.

Summary

The first wave of biotechnology crops, soybean and maize chief among them, were designed with agricultural traits that benefit the US farmer, e.g., herbicide tolerance and insect resistance. Over 80% of soybeans planted in the US last year were genetically enhanced. The second wave of genetically engineered crops will likely focus on traits that are directly beneficially to the consumer, the so-called 'value added traits'. This is perhaps an unprecedented time in US history where the issues of health care costs and the health profile of the US public at large is so much on the minds of the population. The average consumer is aware of issues ranging from the growing cost of medications of the aging baby boomers to trans fats in the cookies and chocolate milk served in elementary school lunchrooms. The educated consumer will increasingly search for food items that provide an additional health benefit. Given the ubiquitous nature of soybean oil in American diets, a healthier oil profile should be welcomed by most consumers. Research focused on engineering functional soybean food will also aid US farmers to meet consumer demand for healthier food items, while allowing them to continue to profit and compete in both domestic and international markets.

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