Nutritional and Safety Assessments of Foods and Feeds Nutritionally Improved through Biotechnology: Case Studies

Prepared by a Task Force of the ILSI International Food Biotechnology Committee

COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

ILSI International Life Sciences INSTITUTE

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By bringing together scientists from academia, government, industry, and the public sector, ILSI seeks a balanced approach to solving problems of common concern for the well-being of the general public.

ILSI is headquartered in Washington, D.C. ILSI branches include Argentina, Brazil, Europe, India, Japan, Korea, Mexico, North Africa and Gulf Region, North America, North Andean, South Africa, South Andean, Southeast Asia Region, the Focal Point in China, and the ILSI Health and Environmental Sciences Inst. ILSI also accomplishes its work through the ILSI Research Foundation (composed of the ILSI Human Nutrition Inst., the ILSI Risk Science Inst. and the ILSI Center for Health Promotion). ILSI receives financial support from industry, government, and foundations.

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Executive Summary

During the last 2 decades, the public and private sectors have made substantial international research progress toward improving the nutritional value of a wide range of food and feed crops. Nevertheless, significant numbers of people still suffer from the effects of undernutrition. In addition, the nutritional quality of feed is often a limiting factor in livestock production systems, particularly those in developing countries. As newly developed crops with nutritionally improved traits come closer to being available to producers and consumers, we must ensure that scientifically sound and efficient processes are used to assess the safety and nutritional quality of these crops. Such processes will facilitate deploying these crops to those world areas with large numbers of people who need them. This document describes 5 case studies of crops with improved nutritional value. These case studies examine the principles and recommendations published by the Intl. Life Sciences Ins. (ILSI) in 2004 for the safety and nutritional assessment of foods and feeds derived from nutritionally improved crops (ILSI 2004). One overarching conclusion that spans all 5 case studies is that the comparative safety assessment process is a valid approach. Such a process has been endorsed by many publications and organizations, including the 2004 ILSI publication. The type and extent of data that are appropriate for a scientifically sound comparative safety assessment are presented on a case-by-case basis in a manner that takes into account scientific results published since the 2004 ILSI report.

Background on the Importance of Nutritionally Improved Foods and Feeds

The United Nations (UN) charter declared that freedom from hunger is a fundamental human right. Diets that are deficient in essential nutrients can be a pervasive cause of hunger and undernutrition. The UN Millennium Project recognized that the number of undernourished people in the world had fallen from approximately 1.5 billion in the early 1970s to around 850 million by the 1990s, and targeted to reduce this number by half by 2015. However, it is sobering to note that even the achievement of this goal will leave the world with over 400 million undernourished humans.

More than 200 million of the world's hungry are children, and at least 5 million of them die each year from undernutrition. Dietary deficiencies take a staggering toll on physical and mental development, which has implications for educational achievement, work performance, and, consequently, economic prospects. Inadequate nutrition also contributes to death from a wide variety of infectious diseases, many of which would not be fatal in well-nourished children.

Insufficient food intake results in many forms of macro- and micronutrient undernutrition. Micronutrient deficiencies are widespread in developing countries. The World Health Organization (WHO) has recognized that such nutrient deficiencies have a catastrophic effect on the health and quality of life of at least 2 billion people. Deficiencies in micronutrients such as iron, vitamin A, iodine, zinc, and folic acid affect large numbers of people, especially children, resulting in significant morbidity and mortality. In many situations, deficiencies in energy and multiple nutrients occur, and these are thought to exert synergistically negative effects. One example is protein-energy malnutrition (PEM), a macronutrient deficiency that is often associated with micronutrient deficiencies. For this reason, most nutritional scientists believe that the long-term solution to under- and malnutrition will be achieved only when all people have access to a balanced, varied, and plentiful diet that meets the known nutritional requirements.

There is no single solution to the complex problem of undernutrition and malnutrition. One approach, undertaken by plant scientists, has been to improve the macro- and micronutrient content of staple crops consumed in developing countries. In addition to using the natural variation present in crop germplasm, modern biotechnology tools are also being used to develop these more nutritious crops. Crops that have been nutritionally enhanced through either modern biotechnology or conventional plant breeding can be thought of as being biofortified. They have inherent fortification in which the level of a nutrient in the crop is enhanced above that normally present. The new varieties developed through modern biotechnology have been described with various terms, including genetically modified (GM or GMO), genetically engineered (GE or GEO), transgenic, biotech, bioengineered, recombinant, and plants with novel traits (PNT). For the present discussion, the term "GM" will be used because of its simplicity and broad recognition.

Research Developments since Publication of the ILSI Document "Safety and Nutritional Assessment of Nutritionally Improved Foods and Feeds"

In 2002, a task force of international scientific experts, convened by the ILSI Intl. Food Biotechnology Committee (IFBiC), addressed the topic of the safety and nutritional assessments of foods and feeds that are nutritionally improved through modern biotechnology. In 2004, the task force's work culminated in the publication of a report that included a series of recommendations for the nutritional and safety assessments of such foods and feeds. This document has gained global recognition from organizations such as the European Food Safety Agency and has been cited by Japan and Australia in 2005 in their comments to Codex Alimentarius. The substantial equivalence paradigm, called the comparative safety assessment process in the 2004 ILSI publication, is a basic principle in the document. This paradigm is one of the topics discussed within the present publication to demonstrate how it can be implemented in the safety assessment process of specific nutritionally improved crops. The comparative safety assessment process is the starting point, not the conclusion, of the analysis. Significant differences in composition are expected to be observed in the case of nutritionally enhanced crops. These differences are intended and should not be considered negative findings because altered composition was the objective of the development process. Instead, the nutritional and safety implications of any potentially significant differences must be assessed on a case-by-case basis.

Since the publication of the 2004 ILSI report, research has progressed on several topics central to the type of data needed to complete a scientifically sound comparative safety assessment of a nutritionally improved food or feed crop. Those topics are presented in the specific case studies in this publication. However, the purpose of reviewing these developments is to present new information that might impact the appropriate form or amount of data for a scientifically sound comparative safety assessment process, while clearly reinforcing the underlying principle of the comparative assessment process that is consistent with the 2004 publication.

One topic that has benefited from new insights is the analysis of genetic changes in GM crops compared to the genetic changes that occurred historically during plant evolution, crop domestication, and the many forms of "conventional breeding." It is relevant to the discussion of the present case studies of nutritionally improved crops because 1 crop was developed through conventional breeding practices, while the other 4 were developed using modern biotechnology. Conventional breeding also provides a baseline that is required for the comparative approach. Conventional crops (that is, crops produced as a consequence of traditional breeding, natural selection) might be casually described as "natural" or characterized as unchanged over time, while GM crops are typically described as products of human intervention. However, human intervention has been as critical to agronomically viable, nutritious, safe, and palatable food and feed crops developed through conventional crop domestication and breeding practices as it has been for GM crops. The goal of domestication is to produce crops with uniformity and desirable agronomic traits, and not necessarily to have plants with increased fitness. In fact, recent evidence on the nature of the changes that occur during conventional plant breeding challenges this perception. The nature of the genetic changes to a plant species brought about by domestication and breeding can be larger in scale and less well defined than the genetic changes to a species that arise from the application of modern biotechnology. For example, it has been stated that "the occurrence of unintended effects is not unique to the application of recDNA techniques, but also occurs frequently in conventional breeding" (Kuiper and others 2001) and that "in fact, conventional breeding programs generally evaluate populations with much wider ranges of phenotypic variation than is observed in transgenic programs" (Bradford and others 2005b). The plants used as major food crops today that were produced by conventional breeding have changed more quickly (for example, over the past few hundred years) than would have been possible through "natural" evolution without human intervention. Furthermore, the changes are commonly in the opposite direction of Darwinian "survival of the fittest." Natural selection creates resilient biological systems with properties that adapt to a variety of environmental conditions and ensures continuation of the species. Unlike natural selection, conventional plant breeding and domestication of many crops often create gene combinations that would not survive without ongoing human intervention.

In addition, it has become clear that major domesticated crops have a wide genetic diversity that reflects the various global environments in which they are grown, and that such diversity is possible because breeding often takes advantage of the hypermutable, genetically fluid characteristics of plant genomes. Extensive variation in DNA content (both amount and makeup) is normal within a species so that members of the same species may possess a slightly different complement of genes or display heterogeneity within specific genes. DNA rearrangements and mutations are common, natural phenomena, and can result in the loss of some proteins, the modification of others, and the creation of novel proteins. Scientific experts sponsored by organizations such as the Food and Agriculture Organization of the United Nations (FAO), the European Commission (EC), and the U.S. Natl. Academy of Sciences (NAS) have frequently studied variations due to breeding and modern biotechnology application. In each case, they concluded that modern biotechnology is no more likely to produce unintended effects than conventional breeding is. Indeed, many expert reviews concluded that the more defined nature of the changes introduced into crops via modern biotechnology may actually be safer than changes produced by conventional plant breeding.

For example, it has been demonstrated that insertion of an entire high-flux pathway into GM *Arabidopsis* plants was achievable without pleiotropic side effects when assessed by combined analyses of the morphological phenotypes or through metabolic and transcript profiling (Kristensen and others 2005). Similarly, extensive analytical comparison of conventional wheat to GM wheat expressing a high molecular weight subunit of the HMW subunit of gluten proteins showed that the expression of the transgene in the GM lines was as that of the corresponding endogenous genes, the GM and control lines showed similar stability in agronomic performance and grain functional properties when grown at multiple sites and years, the gene expression profiles in developing grains of GM and control lines are much more similar to those of the parental lines than are the profiles of lines produced by conventional plant breeding, and the metabolite profiles of control and GM lines usually fell within the range of variation that is observed between genotypes of the species or samples of the same genotype grown under varying environmental conditions (Shewry and others 2007).

The data currently used for the safety assessment of GM crops have focused on the potential perceived risks associated with modern biotechnology. There are now worldwide data from more than 10 y of commercial use of GM crops and over 2 decades of research experience, and no verified adverse consequences have been reported (James 1996). These results allayed the concerns of many and resulted in greater acceptance of GM foods. Indeed, some scientists have begun to question the painstaking premarket safety assessment of GM crops as practiced in some countries, and recommend that the extent and type of data that are part of a current safety assessment be updated to reflect this extensive safe experience with GM crops, coupled with new information about plant genome plasticity. They suggest that refinements to the process could include incorporating factors such as "familiarity" (for example, for commonly used proteins such as CP4 EPSPS, Cry1Ab, and PAT) and the gene source (for example, when the gene is from the same crop species or is one with a history of safe use) into the overall safety assessment, influencing the extent to which event-specific data are needed.

Recent reports have demonstrated that GM crops are often more closely related to the isogenic parental strain used in their

development than to other members of the same genus and species. For example, metabolomic studies in *Solanum tuberosum* have shown that conventional plant breeding produces both intended and unintended effects and that insertion of transgenes can occur with little apparent effect on composition, even when the GM variety produces significant quantities of a new metabolite (for example, inulin) (Catchpole and others 2005). Indeed, when the metabolites (DP2-3 fructans) that accumulate because of the presence of the introduced gene and its expressed product were removed from the analysis parameters, multivariate statistical analysis showed no significant variation in the metabolic phenotype, including harmful glycoalkaloids, between the GM crop and the progenitor lines; whereas conventionally bred cultivars showed clearly separated metabolic phenotypes. Similar results have been observed at the level of the proteome (for example, the set of proteins expressed by an organism's genome) for other plant species.

In the 2004 ILSI report, it was suggested that it is unwise to set a fixed numeric limit to the variation permitted for a metabolite as a trigger for further safety assessment. It was instead recommended that limits should depend on the specific metabolite, its role in safety and nutrition, and the dietary pattern of consumption in the target population, thus making an arbitrary limit across all composition analytes of little value. Nonetheless, there continues to be misunderstanding about the comparative safety assessment process. Substantial equivalence is not a conclusion to be reached, but rather the starting point for comparing a novel product to something with a history of safe use, in which the identified differences are subjected to additional assessment. Attempting to demonstrate the absence of any statistically significant differences between the new crop and its conventional counterpart will clearly be futile for crops whose compositions have been deliberately altered. For the major commodity crops (for example, maize, soy), the content of nutrients, antinutrients, and toxicants is well known and has a long history. This enabled the Organisation for Economic Co-operation and Development (OECD) to establish a well-defined list of constituents that should be assessed for several of these major crops, even if some of these constituents are not nutritionally or toxicologically important. Major crops are frequently, but not always, chosen as the target for nutritional enhancement, and these OECD composition guidelines are appropriate for improved nutrition varieties of these major crops. It is important to regard nutritional changes in the context of that food's consumption by various groups in the population and its contribution relative to specific nutrients—obviously few foods are good sources of all, or even many, nutrients. Similarly, targeted analysis of the well-understood antinutrients and natural toxicants in the major crops humans and animals consume will reveal whether unacceptable changes have occurred that would warrant safety concerns.

Several case studies in this document involve introduction of a protein not currently present in the crop. Therefore, it is important to note that another ILSI IFBiC task force is developing the scientific basis and recommendation for a framework for the safety assessment of proteins. The report from this Protein Safety Task Force, which is expected to be published in 2008, will describe the characteristics of proteins and how such characteristics should drive the safety assessment. It will include recommendations for a tiered, weight-of-evidence approach to the safety assessment of proteins.

Within the 2004 ILSI publication, comprehensive, untargeted compositional analysis techniques, such as metabolomics, proteomics, and transcriptomics, were suggested as potentially useful tools to screen for unintended changes in food and feed crops. Efforts continue in these areas to standardize the reporting structure of such "–omics" data and to recommend current best practices. These are important steps to harmonize workflows and to enable queries of the metabolomes, proteomes, or transcriptomes of novel foods against databases in order to find meaningful unintended and unexpected events. However, to date, public repositories on baseline metabolomes, proteomes, and transcriptomes of crops (such as is available for composition data at www.cropcomposition.org) are just becoming available, and it will require substantial time and financial commitment to establish and maintain databases that are standardized, validated, and monitored. It is recommended that data from analyses of samples from different environmental conditions be represented within crop profiling databases to enable baseline assessments to which profiles of metabolites and proteins in novel foods may be compared, if deemed necessary.

At the time of the 2004 ILSI publication, many studies were in progress to determine if DNA or protein from GM crops could be detected in products from animals fed these products. Two recent reports reviewed publications of studies conducted in a range of livestock species (Flachowsky and others 2005; Phipps and others 2006). Fragments of DNA from multicopy endogenous genes were found at trace levels in specific tissues in some of the studies in this review, although these fragments were typically very small, and certainly too small to encode a functional gene. No fragments of transgenic DNA that could retain any biological activity were detected in any animal-derived products. These reviews of published reports, plus other publications, demonstrate that to date there is still no scientific evidence to suggest that milk, meat, and eggs derived from animals consuming GM crops are anything other than as safe as those derived from animals fed conventional crops.

Application of Risk Analysis to Improved Nutrition Crops

In the Codex Alimentarius model, risk analysis is composed of 3 elements (that is, risk assessment, risk management, and risk communication). During risk analysis, the risks are to be weighed against other issues, such as the benefits, with the aim to ensure the highest appropriate level of public health protection and to strive for risk management transparency and continuous communication between assessors and managers during the process. Implementation should be examined and reviewed for its effectiveness in protecting public health. The case studies of nutritionally improved crops in this document focus on recommended scientific assessments of possible risks associated with the new nutritionally improved food or feed. However, science-based premarket assessments, as currently performed, typically do not balance the assessment of potential risks with the intended benefits that accrue from use. For nutritionally enhanced crops, it is particularly important to balance the intended nutritional benefits (for example, improved health,

decreased incidence of disease, suffering, and/or death) against the outcome of the risk characterization. The perceived hazards often represent relatively small risks, whereas the potential nutritional benefits are relatively large. For example, it has been estimated that the development of iron- and zinc-dense varieties of rice and wheat for India and Bangladesh could prevent 44 million cases of anemia over 10 y (Bouis 2002).

Some scientists have begun to ask if the premarket safety assessment used in many countries is attempting to achieve a standard of absolute safety by continually adding data requirements as newer analytical methods come available (Bradford and others 2005a, 2005b). These scientists suggest that, from a scientific perspective, the cumulative experience over a few decades of assessing GM crop safety should allow us to determine which tests need to be applied to new GM varieties to determine if they are as safe as their traditional counterparts with a history of safe consumption. This process is relatively simple for some crop species, while others may require more extensive analysis—yet today, we subject all crop species to the same assessment process, regardless of potential risk. The fundamental concept of the comparative risk assessment process is that it enables a reasonable certainty that a new GM variety of a crop is as safe as the conventional varieties currently being safely consumed by humans and animals.

Case Studies

This publication applies the recommended principles for safety and nutritional assessments of nutritionally enhanced crops set forth in the 2004 ILSI publication to 5 examples of nutritionally enhanced foods and feeds. The case studies are used to illustrate how the 2004 recommendations provide a strong and robust paradigm for safety assessment for "real world" examples of improved nutrition crops. These case studies are at different stages of development; therefore they have differing levels of available data. Nevertheless, the recommendations can be drawn equally from the 2004 ILSI publication. Four of the 5 case studies represent crops in which the nutritional improvement was achieved through application of modern biotechnology (that is, recombinant DNA techniques), and 1 case study reviews a crop improved through conventional breeding. One case study examines a crop primarily used for animal feed, while the other four are examples of crops modified to improve human nutrition.

Food

The 4 food case studies illustrate the variety of improved nutrition crops that are currently being developed to address the dietary insufficiency for particular nutrients in specific locales.

Nearly 70% of the world's population relies on cereal grains as a dietary staple (FAOSTAT 2004). Rice is recognized as the most important cereal for human nutrition, providing over 30% of the energy intake of the population of Asia. Maize ranks third, after rice and wheat, as one of the world's 3 leading food crops. Although maize grain is primarily used for livestock feed in developed countries, maize is a dietary staple in Latin America and Africa. Other crops, such as sweetpotato, are also important secondary staple foods for large numbers of people in Eastern and Southern Africa, especially for subsistence farmers. Sweetpotato is also an important component of animal feed in China. Most crops, and hence the food derived from those crops, are deficient in one or more essential nutrients, and the diets of many in developing countries that rely on these crops lack dietary diversity. A lack of dietary diversity directly increases the risk of nutrient deficiencies.

The 1st case study of nutritionally improved food crops targets increasing maize's nutritional characteristics for human consumption and livestock feeding systems by increasing the level and quality of key nutrients such as protein and oil; this has been a long-term goal of conventional plant breeding. This case study describes "Double-Embryo Maize," a variety being developed through modern biotechnology in which the grain contains 2 embryos, resulting in maize grain with higher protein and oil contents.

The next 2 case studies describe and compare 2 different nutritional improvements of "Sweetpotato." Sweetpotato tuberous roots vary in color (white-, yellow-, orange-, red-, or purple-fleshed), with orange-fleshed types being particularly rich in β-carotene. The 2nd case study involves conventional breeding and selection of orange-fleshed sweetpotato as a crop biofortified with β-carotene to control vitamin A deficiency (VAD). The 3rd case study is based on the recognition that both the protein content and quality of sweetpotato are relatively low, such that improved protein content and quality through biotechnology could benefit animal feed and high sweetpotato-consuming populations at risk for protein-energy malnutrition (PEM).

The 4th case study is "Golden Rice 2," in which the concentration of the most important provitamin A carotenoid, β-carotene, was increased through modern biotechnology to address VAD. It is estimated that 70 g (1/3 cup) of Golden Rice 2 may provide 2/3 of the daily recommended intake of vitamin A for a preschool child, therefore holding great promise to reduce VAD in developing counties.

Feed

Many nutritional limitations to livestock production are present in both developed and developing countries. For nonruminant livestock production systems, maize grain is often the preferred energy source; however, it is low in the essential amino acid lysine. Consequently, diets based on maize must be supplemented with lysine, either from crystalline lysine or from high-protein ingredients (for example, soybean meal, fish meal). The last case study discusses "Lysine maize," a crop in which the lysine content of maize has been increased through biotechnology, making it possible to simplify diet preparation by reducing or even eliminating the addition of crystalline lysine or high-protein supplements to some nonruminant diets.

Conclusions

The crops being developed to improve human or animal nutrition hold great promise in helping to address global nutrition needs. The present examination of 5 case studies (4 of which are GM crops) has reinforced the conclusion that the existing comprehensive safety and nutritional assessment processes used to assess the safety of GM foods and feeds

already introduced into the marketplace are appropriate to ensure the safety and nutritional value of nutritionally improved crops. Additional studies may be needed, on a case-by-case basis, to assess potential safety or nutritional consequences resulting from changed levels of the improved nutritional factor(s). For both conventional and GM crops, the precommercial breeding and development process (for example, selecting a single commercial variety from large numbers of crosses between conventional lines or from hundreds to thousands of initial transformation events for GM crops) eliminates the vast majority of conventionally bred varieties and GM events that contain unintended changes, winnowing down to a single commercialized variety or GM event. In addition, the selected commercial product candidate typically undergoes detailed phenotypic, agronomic, morphological, and compositional analyses to further screen for unintended effects that would limit commercial acceptance or product safety.

Recommendations

The current comparative safety assessment process provides assurance of safety and nutritional quality by identifying similarities and differences between the new food or feed crop and a conventional counterpart with a history of safe use. The similarities noted through this process are not subject to further assessment. The identified differences then become the focus of additional scientific assessment. A number of recommendations resulting from the safety and nutritional assessment of the 5 case studies presented in this document are consistent across all case studies. These consistently noted recommendations are listed below and confirm that the principles set forth in this task force's 2004 ILSI publication are sound.

Recommendation 1. The safety assessment of a nutritionally improved food or feed begins with a comparative assessment of the new food or feed crop with an appropriate comparator crop that has a history of safe use (the definition history of safe use is explored in a recent publication of the ILSI Europe Novel Foods Task Force).

Recommendation 2. To evaluate the safety and nutritional impact of nutritionally improved food and feed crops, it is necessary to develop data on a case-by-case basis in the context of the proposed use of the product in the diet and consequent dietary exposure.

Recommendation 3. The safety of any novel protein(s) introduced into a crop needs to be assessed. It is noted that another ILSI IFBiC publication recommends a tiered, weight-of-evidence approach for the safety assessment of transgenic proteins.

Recommendation 4. Compositional analysis of crops with known toxicants and antinutrient compounds should include analysis of those specific analytes. If warranted, an evaluation of the targeted metabolic pathway should also be conducted to identify specific metabolites for inclusion in the compositional analysis due to safety and/or nutritional considerations.

Recommendation 5. The appropriate phenotypic properties of the nutritionally improved crop need to be assessed when grown in representative production locations as part of the overall comparative safety assessment process. Further study is warranted if significant unintended and unexplainable differences between the improved crop and an appropriate comparator are identified.

Recommendation 6. Studies with laboratory animals can confirm observations from other components of the safety assessment, thereby providing a sense of added safety assurance, although they may lack the sensitivity to reveal unintended minor changes.

Recommendation 7. While feeding studies with target livestock species are not part of the safety assessment, they are important to demonstrate the expected nutritional benefit of nutritionally enhanced feed crops.

Recommendation 8. Premarket studies in humans might be appropriate on a case-by-case basis to assess the nutritional effectiveness of the improved nutrition crop in those cases where alteration by conventional breeding would trigger similar studies.

Recommendation 9. Premarket assessment regarding the impact of the introduction of an improved nutrition crop on the nutrient intake of consumers may often be appropriate (for example, when changes in agricultural practices or changes in consumer-led dietary intakes are anticipated).

Recommendation 10. The scientific assessment of the possible consequences of the adoption of improved nutrition crops should balance not only assessing the potential risks, but also considering the opportunity for benefits to alleviate undernutrition for a potentially large number of people. In this regard, it may be useful to think of benefits as the removal of the risk of harm caused by nutritional deficiencies. This will provide the relevant data required for a meaningful risk-benefit analysis.

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Chapter 1: Background and Introduction to Case Studies

1.1 Background

During the last decade, the planted area of GM crops modified for agronomic input traits such as herbicide tolerance (*Ht*) and insect resistance (*Bt* is the most common example) has increased from 2 to 102 million ha, and these crops are now grown by over 10 million farmers in a total of 22 developed and developing countries (Brookes and Barfoot 2006; James 2006). Although further expansion in the planted area of these crops is expected, there is increasing interest in the next generation of GM crops, which are those modified for improved nutritional characteristics of feed and food. Table 1–1 illustrates the breadth of international research efforts by both public and private sectors toward improving the nutritional characteristics of a wide range of crops. The reasons for this are easy to understand. In spite of noteworthy progress during the last 2 decades, significant numbers of people still suffer from the effects of mal- and undernutrition. Also, feed quality is often a limiting factor in livestock production systems, particularly those in developing countries.

1.2 Global Undernutrition

The United Nations (UN) charter declared that freedom from hunger is a fundamental human right, and a diet that is deficient in essential nutrients is a pervasive form of hunger. To address persistent and widespread undernutrition punctuated by episodic famines, the First World Food Conference was held in Rome in 1974 with the hope of developing a strategy to eliminate hunger. UN Millennium Project recognized that the number of undernourished people in the world had fallen from approximately 1.5 billion in the early 1970s to around 850 million by the 1990s. With Goal 1, titled "Eradicate Extreme Poverty and Hunger," the Millennium Project set a target to "halve, between 1990 and 2015, the proportion of people who suffer from hunger." However, it is sobering that even the achievement of this goal will still leave the world with over 400 million undernourished humans.

More than 200 million of the world's hungry are children, and at least 5 million die each year from undernutrition. Nutritional deficiencies take a sizeable toll on physical and mental development, which has implications for educational achievement, work performance, and, consequently, economic prospects. Inadequate nutrition also contributes to death from a wide variety of infectious conditions such as diarrhea and respiratory tract infections, many of which would not be fatal in well-nourished children (UN Millennium Project 2005).

Insufficient daily food intake can result in protein-energy malnutrition (PEM, see Appendix 2), which may also be associated with micronutrient deficiencies and disease. Iron deficiency alone affects some 2 billion people, and vitamin A deficiency affects at least 100 million children each year, of whom 1.3 to 2.5 million will die and over 250,000 others will suffer permanent damage such as blindness. Nearly 30% of the world's population is at risk of iodine deficiency. Approximately 800 million people consume iodine-deficient diets, of which 50 million people suffer from iodine deficiency-mediated brain damage. Folic acid deficiency is associated with neural tube defects such as spina bifida, and 250,000 babies with such defects are born each year. Zinc deficiency often accompanies poverty and inadequate diet. Although not as well quantified, the number affected is estimated to be in the same order of magnitude as those with iron deficiency, as the causes are similar. Zinc deficiency clearly diminishes appetite and adversely affects the immune response, both of which affect growth and development. Anemia has also been associated with zinc deficiency. In many situations, multiple deficiencies occur and these are thought to exert synergistically deleterious nutritional effects. For this reason, most nutritional scientists believe that the long-term solution to mal- and undernutrition will be achieved only when all people have access to a balanced, varied, and plentiful diet that meets or exceeds all known human nutritional requirements.

The causes of mal- and undernutrition are complex. If attained, the UN Millennium Project goals (focused on economic development, opportunity, education, and investment as engines to lift people out of poverty) will undoubtedly do much to alleviate undernutrition. Until those goals are achieved, food aid and a variety of micronutrient fortification and pharmaceutical supplementation programs seek to fill the gap in the world's need. Although these strategies may be appropriate for a large number of the undernourished, approximately half of the world's poor are subsistence farmers or small producers who do not buy food on a regular basis. Additionally, pharmaceutical supplementation programs require a well-functioning healthcare system that does not always exist. Thus, fortification and pharmaceutical supplementation programs have not proven fully successful for these individuals, and improvements in crop yield, distribution, and nutritional quality may be a beneficial and appropriate solution in such instances.

In recent years, plant scientists have worked to improve the macro- and micronutrient content of staple crops consumed in developing countries. Besides using the natural variation present in crop germplasm, modern biotechnology tools are also used to construct these more nutritious crops. Crops that have been nutritionally enhanced through either modern biotechnology or conventional plant breeding can be thought of as being biofortified (inherent fortification in which the concentration of nutrient in the crop is enhanced above that normally present).

1.3 Earlier Work

In 2002, the International Intl. Food Biotechnology Committee (IFBiC) of the International Intl. Life Sciences Institute Inst. (ILSI) convened a task force of international scientific experts to address the topic of safety and nutritional

Table 1-1— Examples of crops in research and/or development with nutritionally improved traits intended to provide health benefits to consumers and animals.[a]

Trait	Crop (trait detail)	Reference
Protein and amino acids		
Protein quality and level	Bahiagrass (protein↑)	Luciani and others (2005)
	Canola (amino acid composition)	Roesler and others (1997)
	Maize (amino acid composition; protein↑)	Cromwell and others (1967, 1969), Yang and others (2002), O'Quinn and others (2000), Young and others (2004)
	Potato (amino acid composition; protein↑)	Chakraborty and others (2000), Li and others (2001), Yu and Ao (1997), Atanassov and others (2004)
	Rice (protein↑; amino acid composition)	Katsube and others (1999)
	Soybean (amino acid balance)	Rapp (2002), Dinkins and others (2001)
	Sweetpotato (protein↑)	Prakash and others (2000)
Essential amino acids	Canola (lysine↑)	Falco and others (1995)
	Lupin (methionine↑)	White and others (2001)
	Maize (lysine↑; methionine↑)	Agbios (2006), Lai and Messing (2002)
	Potato (methionine↑)	Zeh and others (2001)
	Sorghum (lysine↑)	Zhao and others (2003)
	Soybean (lysine↑; tryptophan↑)	Falco and others (1995), Galili and others (2002)
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↑)	Del Vecchio (1996), James and others (2003), Dehesh and others (1996), Agbios (2006), Roesler and others (1997)
	Cotton (oleic acid↑; oleic acid + stearic acid↑)	Chapman and others (2001), Liu and others (2002)
	Linseed (+ ω-3 and- 6 fatty acids)	Abbadi and others (2004)
	Maize (oil↑)	Young and others (2004)
	Oil palm (oleic acid↑ or stearic acid↑; oleic acid↑ + palmitic acid↓)	Parveez (2003), Jalani and others (1997)
	Rapeseed (oil and fatty acyl composition)	Zou and others (1997)
	Rice (α-linolenic acid↑)	Anai and others (2003)
	Soybean (oleic acid↑; γ-linolenic acid↑)	Kinney and Knowlton (1998), Reddy and Thomas (1996)
Carbohydrates		
Fructans	Chicory (fructan↑; fructan modification)	Smeekens (1997), Sprenger and others (1997)
	Maize (fructan↑)	Caimi and others (1996)
	Potato (fructan↑)	Hellwege and others (1997)
	Sugar beet (fructan↑)	Smeekens (1997)
Inulin	Potato (inulin↑)	Hellwege and others (2000)
Starch	Rice	Chiang and others (2005)
Micronutrients and functional metabolites		
Vitamins and carotenoids	Canola (vitamin E↑)	Shintani and DellaPenna (1998)

Table 1-1 continued.

Trait	Crop (trait detail)	Reference
	Maize (vitamin E↑; vitamin C↑)	Rocheford and others (2002), Cahoon and others (2003), Chen and others (2003)
	Mustard (+β-carotene)	Shewmaker and others (1999)
	Potato (β-carotene and lutein↑)	Ducreux and others (2005)
	Rice (+ β-carotene)	Ye and others (2000)
	Strawberry (vitamin C↑)	Agius and others (2003)
	Tomato (folate↑; phytoene and β-carotene↑; lycopene≠; provitamin A↑)	Díaz de la Garza and others (2004), Enfissi and others (2005), Mehta and others (2002), Fraser and others (2001), Rosati and others (2000)
Functional secondary metabolites	Apple (+stilbenes)	Szankowski and others (2003)
	Alfalfa (+resveratrol)	Hipskind and Paiva (2000)
	Canola (indole glucosinolate↓)	Chavadej and others (1994)
	Cassava (cyanogen↓)	Siritunga and Sayre (2003)
	Coffee (caffeine↓)	Moisyadi and others (1998), Kato and others (2000), Ogita and others (2003)
	Kiwi (+resveratrol)	Kobayashi and others (2000)
	Maize (flavonoids↑)	Yu and others (2000)
	Potato (steroidal glycoalkaloids↓, anthocyanin and alkaloid glycoside↓; solanin↓)	McCue and others (2003), Lukaszewicz and others (2004)
	Rice (flavonoids↑; +resveratrol)	Shin and others (2006), Stark-Lorenzen and others (1997)
	Soybean (flavonoids↑)	Yu and others (2003)
	Tea (caffeine↓)	Kato and others (2000)
	Tomato (+resveratrol; chlorogenic acid↑; flavonoids↑; stilbene↑)	Giovinazzo and others (2005), Niggeweg and others (2004), Muir and others (2001), Rosati and others (2000)
	Wheat (caffeic and ferulic acids↑; +resveratrol)	UPI (2002)
Mineral availabilities	Alfalfa (phytase↑)	Austin-Phillips and others (1999)
	Lettuce (iron↑)	Goto and others (2000)
	Rice (iron↑)	Lucca and others (2002)
	Soybean (phytase↑)	Denbow and others (1998)
	Wheat (phytase↑)	Brinch-Pedersen and others (2000)

[a]This table excludes protein functionality, starch functionality, fruit ripening/shelf life, taste/aesthetics, and fiber quality traits. It also excludes food quality benefits that are not directly linked to nutrition, such as reduced allergenicity and reduced mycotoxins.

assessments of foods and feeds that are nutritionally improved through modern biotechnology. The resultant publication (ILSI 2004a, 2004b) reported on the principles and concepts proposed for use in this process and produced a series of recommendations for the nutritional and safety assessments of nutritionally improved foods and feeds developed through biotechnology. Other publications have examined the potential costs, benefits, and risks of biotechnology application to agriculture in the developing world, where the majority of agricultural insufficiency and undernutrition occur (Hepple and others 2004).

1.4 Present Study

This publication applies the guidelines and principles recommended for the safety and nutritional assessments of nutritionally enhanced crops in the 2004 ILSI publication to 5 examples of nutritionally enhanced feeds or foods. The case studies are used to illustrate how the 2004 guidelines provide a strong and robust paradigm for safety assessment of "real world" examples of improved nutrition crops. These case studies are at different stages of development, and therefore they have differing levels of available data; however, recommendations

for each case study can be drawn equally from the 2004 ILSI publication.

1.5 Case Studies Included in This Report

1.5.1 Food

The 4 food case studies illustrate the variety of improved nutrition crops that are currently being developed to address the dietary insufficiency for specific nutrients in specific locales, and demonstrate applying the safety assessment principles for nutritionally enhanced foods and feeds outlined in the 2004 ILSI publication.

Cereal grains represent a staple in the diet for nearly 70% of the world population. Rice is recognized as the most important cereal for human nutrition, providing over 30% of the energy intake of the population of Asia. Maize ranks third, after wheat and rice, as one of the world's 3 leading food crops. Although maize grain is primarily used for livestock feed in developed countries, it is a dietary staple in Latin America and Africa. Crops more limited in planted area, such as sweetpotato, are also important secondary staple foods for large numbers of people in Eastern and Southern Africa. Sweetpotato is also an important component of animal feed in China. Most crops, and hence the food derived from those crops, are deficient in one or more essential nutrients. Consequently, a lack of dietary diversity increases the risk of nutrient deficiencies.

Case study 1: Double-embryo maize grain with increased protein and oil content (biotechnology). A long-term goal of conventional plant breeding has been to increase the nutritional characteristics of maize for human consumption and livestock feeding systems by increasing the level and the quality of key nutrients such as protein and oil. The 1st improved nutrition food crop case study describes "Double-Embryo Maize," a variety developed through modern biotechnology in which the grain contains 2 embryos, resulting in maize grain of greatly increased protein and oil contents. This type of maize is still in the relatively early stages of development.

Case studies 2 and 3: Sweetpotato with increased protein content (biotechnology) and sweetpotato with enhanced β-carotene content (conventional breeding). The next 2 case studies describe and compare 2 different nutritional improvements of "Sweetpotato." Sweetpotato tuberous roots vary in color (white-, yellow-, orange-, red-, or purple-fleshed), with orange-fleshed types being particularly rich in β-carotene. The 2nd case study involves conventional breeding and selection of orange-fleshed sweetpotato as a crop biofortified with β-carotene to control vitamin A deficiency (VAD). The 3rd case study is based on the recognition that both the protein content and quality of sweetpotato are relatively low. This case study, therefore, describes using modern biotechnology to increase both the protein content and quality by improving the amino acid profile of the crop to address both PEM and poor quality animal feed.

Case study 4: Rice enriched with β-carotene (biotechnology). Micronutrient deficiencies are widespread in developing countries, and the World Health Organization (WHO) has recognized that such nutrient deficiencies have a catastrophic effect on the health and quality of life of at least 2 billion people. VAD is a global public health challenge, especially where rice is the staple food. It affects about 100 million children; more than 250,000 are severely deficient and become blind each year, and approximately 125,000 of these die within a year (West 2002; WHO 2003). Historically, the emphasis has been on increasing the intake of green leafy vegetables and yellow-orange fruits and vegetables to increase vitamin A intake and on pharmaceutical supplementation with vitamin A, but these strategies have been difficult to implement on a large scale. The need to address VAD is clear and unequivocal. The 4th case study of nutritionally improved food crops is "Golden Rice 2" in which β-carotene, the most important provitamin A carotenoid, was increased using modern biotechnology. It is estimated that 70 g (approximately 1 serving of 1/3 cup rice) of the Golden Rice 2 will provide approximately 2/3 of the daily recommended intake of vitamin A for a preschool child, therefore holding great promise to reduce VAD in developing counties where this crop would be available.

1.5.2 Feed

Nutritional limitations to livestock production are numerous and varied in both developed and developing countries. Animal production is often restricted because feed resources are deficient in one or more specific nutrients (for example, maize grain is typically low in lysine content compared to other crops), have limited nutrient bioavailability (for example, the use of crop residues as the primary forage source in developing countries), or are constrained by the presence of antinutritional factors or toxins (for example, phytate and mycotoxins) (Table 1–1).

Case study 5: Increased lysine content in maize grain (biotechnology). For example, in the case of nonruminant livestock production systems, maize grain is often the preferred energy supplement. However, it is low in the essential amino acid lysine and either a crystalline lysine supplement or a high-protein supplement is required to ensure optimum animal performance. The 5th case study discusses "Lysine maize," a crop in which the lysine content has been increased through biotechnology, making it possible to reduce or even eliminate the addition of crystalline lysine supplements to some nonruminant diets.

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Chapter 2: Recent Developments in the Safety and Nutritional Assessment of Nutritionally Improved Foods and Feeds

2.1 Background

Following the publication of the 2004 ILSI report, research has progressed on several topics central to the completion of a scientifically sound comparative safety assessment of an improved nutrition food or feed crop. Those topics merit review as part of the presentation of the specific case studies in this publication. The purpose for reviewing the topics is to present new information that might affect the form or amount of data appropriate to conduct a scientifically sound comparative safety assessment process, while reinforcing the underlying principle of the comparative assessment process, consistent with the 2004 publication.

2.2 New Insights into Plant Breeding and Unintended Effects

Some scientists have begun to question if the premarket safety assessment used in many countries is attempting to achieve a standard of absolute safety by adding data requirements as newer analytical methods come available (Bradford 2005a, 2005b). Another example is Cellini and others (2004), who concluded:

> The safety assessment of GM crops should focus primarily on the intended novel traits (target gene[s] and product[s]). Unintended effects occur in both GM and non-GM crops; however, GM crops are better characterised. It may be suggested that the two should be treated the same in safety assessments, bearing in mind that safety assessments are not required for non-GM crops.

These scientists suggest that, from a scientific perspective, the cumulative experience of the past 2 decades of assessing GM crop safety should allow us to determine which tests need to be applied to new GM varieties to determine if they are as safe as their traditional counterparts with a history of safe consumption. This process is relatively simple for some crop species, while others may require more extensive analysis—yet today, we subject all crops species to the same assessment process, regardless of risk. The fundamental concept of the comparative risk assessment process is that it enables a reasonable certainty that a new GM variety of a crop is as safe as the conventional varieties currently being safely consumed by humans and animals.

The safety assessment paradigm applied to GM crops attempts to evaluate whether any unintended deleterious changes have occurred (ILSI 2004). This requirement, given that conventionally bred crops are not subjected to comparable premarket safety assessments, may have contributed to the impression that GM crops pose unique risks. Conventional crops are often described as being "natural," with little (if any) changes over millennia, whereas GM crops are sometimes typically described as products of human intervention. Recent studies that challenge this perception provided scientific data on the nature of the changes that occur during plant evolution, crop domestication, and the many forms of "conventional breeding." Therefore, this is one topic that has benefited from new insights and is relevant to this publication of improved nutrition crop case studies.

Most domesticated crops have been developed during the last 5000 y, many within the last few hundred years (Gepts 2002). The nature of the genetic changes to a plant species brought about by domestication and breeding typically is much larger in scale, and much less well-defined, than the genetic changes to a species that arise from modern biotechnology applications. For example, it has been stated that "in fact, conventional breeding programs generally evaluate populations with much wider ranges of phenotypic variation than is observed in transgenic programs…" (Bradford and others 2005b). It is clear from these and other studies (Baker and others 2006; Baudo and others 2006) that the plants used as major food crops have evolved more quickly (for example, over the past few thousand years) than would be possible solely through "natural" evolution, and the changes are often in the opposite direction of Darwinian "survival of the fittest" (Gepts 2002; FAO 2004). Unlike natural selection, which creates resilient biological systems with properties that adapt to a variety of environmental conditions and ensures continuation of the species, conventional plant breeding and crop domestication have often created gene combinations that would rarely survive without human intervention.

Major domesticated crops represent hundreds, or even thousands, of unique varieties cultivated (that is, "cultivars") by humans, each adapted to specific geographic environments used to produce food and feed around the world (Gepts 2002; FAO 2004). This wide diversity in crop varieties is possible, in part, because the plant breeding selection takes advantage of the genetic fluidity of plant genomes, modern maize being an excellent example (Parrott 2006). Extensive variation in DNA content is normal within a species, especially for plants, in which DNA rearrangements are common natural phenomena, and individuals within a plant species can have different numbers of genes through natural selection processes (Cellini and others 2004; Bradford and others 2005a, 2005b). These natural phenomena often come about by random, sequence-independent insertions, deletions, and/or transpositions of DNA, changes that are neither planned nor programmed. On some occasions, genes have also been observed to be transferred between unrelated, sexually incompatible species—with the frequency being sufficient to

say the phenomenon, though not common, is natural (Prins and Zadoks 1994; Ashby and others 1997; Aoki and Syono 1999; Hull and others 2002; Bergthorsson and others 2003, 2004).

Scientific experts sponsored by organizations such as the Food and Agriculture Organization of the United Nations (FAO), the European Commission (EC), and the U.S. Natl. Academy of Sciences (NAS) have studied variations due to breeding and application of modern biotechnology. In each case, their research concluded that modern biotechnology is no more likely than conventional breeding to produce unintended effects (EC 2001; FAO 2004; NRC 2004). It has been stated that "the occurrence of unintended effects is not unique for the application of recDNA techniques, but also occurs frequently in conventional breeding" (Kuiper and others 2001). Indeed, many expert reviews concluded that the greater precision and more defined nature of the changes introduced into crops via modern biotechnology may actually be safer than changes produced by conventional plant breeding. For example, it has been demonstrated that inserting an entire high-flux cyanogenic glucoside pathway into GM *Arabidopsis* plants was achievable without pleiotropic side effects when assessed by combined analyses of the morphological phenotypes and through metabolic and transcript profiling (Kristensen and others 2005).

Some scientists recommend updating the current safety assessment paradigm to reflect nearly 2 decades of experience with GM crops that, coupled with new knowledge about plant genome plasticity, could lead to a more balanced and reasonable safety assessment process (Cellini and others 2004; Bradford and others 2005a, 2005b). Refinements to the process could include incorporating factors such as "familiarity" (for example, for commonly used proteins such as CP4 EPSPS, Cry1Ab, and PAT) and the gene's source (for example, when the gene is from the same crop species, or a crop with a history of safe use) into the overall safety assessment, influencing the extent to which event-specific data are needed.

2.3 An Insight into Substantial Equivalence, Composition, and Dietary Intake

The "substantial equivalence" paradigm, referred to as the "comparative safety assessment process" in the 2004 ILSI publication, is a key principle in the document (ILSI 2004). However, because of continuing misinterpretation of substantial equivalence, further discussion of this principle is warranted and is a key topic of the present publication.

The comparative safety assessment process is the starting point, not the conclusion, of the analysis. The overall safety assessment begins with the concept of a comparative approach that identifies the similarities and differences between the GM product and one or more appropriate comparators with a known history of safe use—a model that is found in all international crop biotechnology assessment guidelines. Consideration is given to the history and safe use of the conventional line that serves as the principal comparator. This ensures that identifying similarities with the comparator provides a solid basis for concluding that these aspects of the product are not likely to raise concerns. Considering the safety of the parent crop and the gene donor helps to eliminate the possibility of potentially undesirable traits being introduced from those sources. Additionally, understanding the safety of the parent crop and gene donor facilitates a directed search in the GM product to determine to what, if any, extent the trait(s) may have been transferred.

If the comparison of the GM product to the comparator identifies differences that include the introduced trait, such differences are subjected to an evaluation of their potential toxic, allergenic, or nutritional impact. Thoroughly evaluating the identified differences from a safety perspective makes possible a conclusion about the safety of food or feed derived from the GM crop, as compared to its traditional counterpart with a history of safe use/consumption. Using this approach, in total more than 100 GM crops have been approved worldwide, with the conclusion reached in each case that foods and feeds derived from these GM crops are as safe and nutritious as those derived from their traditional counterpart crops. The lack of any demonstrable adverse effects resulting from the production and consumption of GM crops grown on more than 1 billion cumulative acres over the last 10+ y supports these safety conclusions. In the case of nutritionally enhanced crops, significant composition differences are intended; therefore they are to be expected. Significant composition differences of nutritionally enhanced crops should not be considered to be negative attributes since many will be intentional and desirable improvements over the conventional counterpart. Instead, the nutritional and safety implications of the potentially significant observed differences must be assessed on a case-by-case basis.

Recent reports have demonstrated that GM crops have a composition more similar to the isogenic parental strain used in their development than that same strain is to other breeding cultivars of the same genus and species. This effect has been observed at the proteome level for *Arabidopsis thaliana* (Ruebelt and others 2006a, 2006b, 2006c), potato (Lehesranta and others 2005), tomato (Corpillo and others 2004), and wheat (Shewry and others 2007). In these studies, 2-dimensional (2-D) gel electrophoresis was used to compare the protein content of conventional and GM varieties. In each case, GM plants were found to be more similar to the parental isogenic variety from which they were derived than the variety developed through conventional breeding was to other conventional varieties of the same species. Parallel results have been observed at the metabolomic level for wheat (Baker and others 2006) and potato (Catchpole and others 2005). Catchpole and coworkers concluded:

> A major finding from the present study was the large variation in metabolite profile between the conventional cultivars…. In the context of substantial equivalence, we show that the metabolite composition of field-grown inulin-producing potatoes were within the natural metabolite range of classical cultivars and were, in fact, very similar to the progenitor line, Désirée, with the exception of the introduced genes….

The results of this study highlight that conventional plant breeding produces both intended and unintended effects. The study also shows that inserting transgenes into a crop can occur with little apparent effect on composition, even when the GM variety produces significant quantities of a new metabolite (for example, inulin in this potato example). Indeed, when the introduced gene product (DP2-3 fructans) was removed from the analysis parameters, multivariate statistical analysis showed no significant variation in the metabolic phenotype, including harmful

glycoalkaloids, between the GM crop and the progenitor lines, whereas other conventionally bred cultivars showed clearly separated metabolic phenotypes. Naturally, such conclusions can only be reached on a case-by-case basis and cannot be generalized to conclude that all GM crops have the same metabolome as their parent strain. This underscores the need for careful compositional analysis of key metabolites, nutrients, antinutrients, natural toxicants, and other metabolites whose content one might expect to be altered by the specific metabolic changes introduced into the new product.

Very recently, a detailed analysis of conventional and GM wheat lines was reported (Shewry and others 2007). Transcriptomics was conducted on glasshouse-grown control and GM wheat plants. In addition, grain samples from control and GM wheat lines grown in replicated field trials over 4 y and from 2 sites with contrasting climates were subjected to a range of measurements, including dry weight, nitrogen content, protein composition by SDS-PAGE, dough mixing properties by Mixograph, and metabolite profiles. These analyses were used to draw conclusions about the relative stability of expression of the endogenous and GM forms of the HMW subunit genes and their impact on the grain composition and properties. The conclusions were: (1) The expression of the transgenes in the lines studied is not intrinsically more or less stable than that of the corresponding endogenous genes. (2) The GM and control lines show similar stability in agronomic performance and grain functional properties when grown at multiple sites and years. (3) The gene expression profiles in developing grains of GM and control lines are much more similar to those of the parental lines than are the profiles of lines produced by conventional plant breeding. (4) The metabolite profiles of control and GM lines usually fall within the range of variation which is observed between genotypes of the species or samples of the same genotype grown under varying environmental conditions. These results clearly demonstrate that it is possible to produce GM wheat lines that are equivalent to conventional wheat at the level of analysis provided by modern ''–omics'' technologies, except for effects that are directly attributable to the desired new GM trait.

Hothorn and Oberdoerfer (2006) described an elegant statistical treatment to analyze each measured analyte, with the authors concluding that further analysis should be undertaken when any analyte of the GM crop is outside of a blanket threshold of ± 20% range. This 20% threshold is also offered as a way to establish substantial equivalence of a new product. For reasons noted previously, substantial equivalence is not the endpoint of a safety assessment, but instead a process through which differences are identified and subjected to additional safety assessment. However, the concept of a blanket threshold for all compositional analytes does not consider the crop's relative importance for each component in terms of overall diet and nutritional status for the population. As mentioned in the 2004 ILSI publication and elsewhere in this document, for any given crop, it is most important to assess the nutrients for which that crop is a significant dietary source. Therefore, the list of compositional analytes requiring close scrutiny must be determined on a crop-by-crop basis, such as has been done by the Organisation for Economic Co-Operation and Development (OECD) consensus documents for major food crops. The proposal that *all* measured components must fall within the ± 20% range is, therefore, not only unnecessary, but is scientifically inappropriate since it is not nutritionally justifiable.

Moreover, if one applies the same standards Hothorne and Oberdoerfer (2006) suggest to traditionally bred crops, the observed large composition variations and recently observed unintended changes that occur during conventional breeding (Cellini and others 2004) would trigger extensive safety testing of these conventional crops. To note, attempting to demonstrate the absence of any statistically significant differences between the new crop and its conventional counterpart will be futile for crops whose compositions have been deliberately altered. For the major commodity crops (for example, maize, soy), the contents of essential nutrients, antinutrients, and toxicants are well known from a long history of study and safe consumption. This has enabled the OECD to establish a well-defined list of constituents that should be assessed for several of these major crops, such as maize (OECD 2003a). Major crops are frequently, but not always, chosen as the target for nutritional enhancement, and these OECD composition guidelines are appropriate for improved nutrition varieties of these major crops. It is important to regard nutritional changes in the context of that food's consumption by various groups in the population and the food's contribution relative to specific nutrients---obviously few foods are good sources of all, or even many, nutrients (Senti and Rizek 1974; Spiher 1974). Similarly, targeted analysis of the well-understood antinutrients and natural toxicants in the major crops humans and animals consume will reveal whether unacceptable changes have occurred that would warrant safety concerns.

The 2004 ILSI report concluded that it is unwise to set a fixed numerical limit to the variation permitted for a metabolite as a trigger for further safety assessment. The report suggested that limits depend on the specific metabolite, its role in safety and nutrition, and the dietary pattern of consumption in the target population. The objective was to avoid measuring every analyte based on blanket criteria, even when changes in the analyte would have no measurable impact on health and/or nutrition. This is in contrast to an earlier approach by the U.S. Food and Drug Administration (FDA) for new varieties of crops developed through conventional breeding (Senti and Rizek 1974; Spiher 1974).

Changes in crop composition are unlikely to have a significant nutritional impact unless they extensively alter the intake and/or bioavailability of an essential nutrient (Bouis 2002; ILSI 2004). The FDA examined this concept as it considered the nutritional and safety consequences of composition variation that result from conventional crop breeding (Senti and Rizek 1974; Spiher 1974). The FDA set a 20% change in any nutritionally important analyte as a trigger that would require further safety studies. The list of crops and nutrients necessary to be considered was pared down to 9 major crop plants that contributed 5% or more to the average dietary intake of Americans at that time, and to a short series of nutrients—those provided in a meaningful quantity by that specific crop. However, a 20% change in a specific nutrient from a food that provides only 5% of that nutrient in the human diet results in a 1% change that is most likely insignificant in terms of the overall dietary intake of that nutrient. A report summarized the levels of each contributed nutrient by each of the major foods in the diet at that time (Senti and Rizek 1974). Since 1974, although the FDA has reserved the authority to regulate crops produced through conventional

breeding, they have not actively enforced the 20% change triggering rule in practice because a long history of plant breeding shows the technology is generally safe. Moreover, consumers often exercise their freedom of food choice in a manner that results in a far greater variability in nutrient intake and overall dietary composition than would be possible from a 20% change in a single nutrient from a single dietary component.

It is reasonable to expect that significant changes in composition will be observed, and it is important to regard such changes in the context of that food's consumption by various groups in the population and its contribution of specific nutrients. Similarly, targeted analysis of the well-understood antinutrients and natural toxicants in the major crops humans and animals consume will reveal whether unacceptable changes have occurred that would warrant safety concerns.

2.4 Recent Developments in Food Protein Safety Assessments

Several of the case studies in this document involve introducing a protein not currently present in the crop. Therefore, it is important to note that another IFBiC task force is developing the scientific basis and recommendation for a framework for the safety assessment of proteins. The report from this Protein Safety Task Force, which is expected to be published in 2008, will describe the characteristics of proteins and how such characteristics should drive the safety assessment. It will include recommendations for a tiered, weight-of-evidence approach to the safety assessment of proteins. The publication will also include a detailed description (including applicability, best practices for use, and limitations) of current assessments to demonstrate protein safety, including expression level and pattern, history of safe use, bioinformatics, mode-of-action, protein stability, and toxicological testing. The definition of history of safe use is explored in a recent publication of the ILSI Europe Novel Foods Task Force (Constable and others, 2007).

2.4.1 Protein safety

Currently, appropriate toxicological studies are typically needed for the introduced protein in GM crops (Codex 2003). The assessment often includes a description of the biological function of the protein in the plant, when known, and studies such as a bioinformatic comparison of the new protein's amino acid sequence with a database of all publicly available protein sequences to determine if the protein is homologous to protein with known toxicity (for example, protease inhibitors, lectins), and testing the newly introduced protein's stability to heat or processing. Appropriate toxicity studies (such as acute toxicity feeding studies in animals) may be needed in cases where the protein present in the food is dissimilar to proteins that have previously been consumed safely in food.

2.4.2 Protein allergenicity

The potential allergenicity of new genetic variants of crops theoretically presents 3 different kinds of concerns (Lehrer and Bannon 2005):

1. that a known protein allergen might be transferred to a crop plant;
2. that the level of endogenous protein allergens (for example, soy allergens) might be increased; and
3. that a novel protein with no prior history of human consumption might be introduced into a crop plant and become an allergen.

A stepwise evaluation scheme, first suggested by ILSI (1996), has been used around the world to ensure that GM crops are no more likely than their conventional counterparts to induce food allergies. No substantiated incidents of food allergy caused by commercialized GM crops have been documented during the 1st decade of their commercial adoption, which is reassuring given the broad acceptance of GM crops by farmers around the world (James 2006). For example, claims that the Cry9C protein caused food allergies could not be authenticated (Lehrer and Bannon 2005). The absence of allergenicity should not be surprising, however, because all GM crops introduced into the marketplace for food and feed usage have been subjected to a thorough premarket allergenic potential assessment. Additionally, it is becoming clear that very few families of proteins have the potential to induce food allergy when presented in a food matrix (Jenkins and others 2005).

It is relatively straightforward to assess the allergenic potential of an introduced protein from a plant with a known allergen, as exemplified by the successful identification of an allergen from Brazil nut, expressed in soybeans (Nordlee and others 1996). By comparison, a robust set of analyses is required to determine whether novel food proteins pose a concern for allergenicity. Food allergies depend not only on the allergen itself, but also on host genetics and physiological status, food use/avoidance behavior, and environmental factors.

The molecular properties of a protein should be considered when assessing potential allergenicity (Breiteneder and Mills 2005). Recent studies show that most food allergens fall into one of a few specific protein superfamilies (Mills and others 2004; Breiteneder and Mills 2005). Bioinformatic analyses can be effective in determining the level of similarity between protein sequences when examining allergenic potential. For example, if a protein is not a member of the known food allergen protein families and has a low level of sequence similarity to known allergens, it is unlikely that it will be allergenic. Bioinformatic analyses are recommended as part of the most recent FAO/WHO consensus (Codex 2003). The amino acid sequences of proteins introduced into GM crops are screened by bioinformatic analysis for similarity to sequences of known allergens. A query sequence is considered to possess an immunologically significant sequence if it has a sequence identity with a known or putative allergen of at least 8 linearly contiguous amino acids, the smallest likely IgE binding epitope (Metcalfe and others 1996; Hileman and others 2002).

However, the lack of value for sequence matches for less than 8 amino acids (for example, a 6 or 7 amino acid sliding-window) was recently demonstrated in a comprehensive analysis of short peptide match frequencies (Silvanovich and others 2006). The suggestion to increase bioinformatic search stringency by examining 6 amino acid residue matches between allergens and novel proteins (FAO 2001) has been shown to not be scientifically reasonable because of high rates of false positive, random associations (Bannon and Ogawa 2006). In addition, no further margin of confidence in identifying proteins with cross-reactivity

to allergens was shown from this approach. Nevertheless, there are opportunities to develop increasingly useful bioinformatic strategies. These may include constructing allergen epitope databases or using conformational protein structures to identify potential allergens (Thomas and others 2004; Bannon and Ogawa 2006).

Some crops, such as soya, are known to contain endogenous allergenic proteins; therefore another potential concern is that introducing new DNA into a crop may alter the level of one or more of these endogenous allergenic proteins. To date, however, GM varieties of crops such as soya have shown no evidence of possessing increased allergenicity due to undergoing transformation (Park and others 2001; Batista and others 2005).

Allergy safety assessments use a panel of characteristics to evaluate novel proteins for allergenic potential. If a novel food protein is not similar to allergens and is not derived from allergenic protein families, it is unlikely to provoke allergy. Moreover, if a protein is degraded in simulated gastric fluid, is small in size (for example, < 10 kDa), and is not glycosylated, the likelihood that it will be an allergen is also unlikely (Lehrer and Bannon 2005; Sánchez-Monge and Salcedo 2005). A major research challenge is the development of methods to predict the allergenic potential of novel food proteins that have no relationship to known allergens. Animal models offer some potential for measuring allergenicity. The predictive capability of animals is complicated, however, by the absence of scientifically justified model systems (McClain and Bannon 2006). Several animal model systems (for example, canine, murine, and porcine) are being researched (Lehrer and Bannon 2005; Sánchez-Monge and Salcedo 2005). Other methods of measuring allergenic potential, such as in vitro IgE binding in the sera of allergic patients, are currently under review for their utility in allergy safety assessments (Thomas and others 2006).

There are many factors that may elicit food protein allergy, including extrinsic factors such as food use and processing; however, this is not well understood and is a continuing topic of research and discussion (ILSI-HESI 2006). It is important to note that many proteins possess one or more of the properties characteristic of an allergen, and yet they do not cause allergy. The likelihood that a novel biotechnology-derived protein will be an allergen is very low for proteins without sequence similarity and physical properties comparable to known allergens. From a safety perspective, the risk of allergy due to introduction of a novel protein into a crop remains low, regardless of whether proteins are introduced through conventional crop breeding or modern biotechnology.

2.5 Developments in Nontargeted "Profiling" Composition Analysis Techniques

Comprehensive, untargeted profiling techniques, such as metabolomics, proteomics, and transcriptomics, were discussed in chapter 6 of the 2004 ILSI publication as potentially useful tools to screen for unintended changes in the edible parts of crops (ILSI 2004). Efforts continue in these areas to standardize the reporting structure of such "–omics" data and to recommend current best practices. These are important steps to harmonize workflows and to enable queries of the metabolomes, proteomes, or transcriptomes of novel foods against databases in order to find meaningful unintended and unexpected events. However, to date, there are no public repositories on baseline metabolomes, proteomes, and transcriptomes of crops (such as that for composition data at www.cropcomposition.org), and it will require substantial time and financial commitment to establish and maintain databases that are standardized, validated, and monitored. It is recommended that data from analyses of relevant tissue samples from different environmental conditions be represented within crop profiling databases to provide baseline assessments to which profiles of metabolites and proteins in novel foods may be compared.

2.5.1 Metabolomics

Metabolomics, which shares the same etymological root as metabolism (metabol, GK. change), seeks to understand changes in biological systems in terms of changes in metabolism. A major aspiration is the identification and semiquantification of all metabolites (often termed metabolic profiling) in a given biological context (Fiehn and others 2005). This represents a demanding challenge, for which sophisticated analytical methods such as mass spectrometry (MS) coupled to gas and liquid chromatography (GC/MS and LC/MS) are generally employed. Although the ideal metabolomics methodology would generate, as nearly as possible, a quantitative global analysis of metabolite content and of metabolic flux, current metabolic profiling methods (for example, GC/MS, LC/MS) allow identification of only a subset of previously described metabolites. Furthermore, accurate quantitation in nontargeted metabolic profiling is often not possible due to both coelution and differential detector responses of metabolite analytes.

To date, studies have been restricted to single, relatively small batches of plants produced under controlled growth conditions. Many such studies adopt a 2-tiered, hierarchical profiling approach. For an initial assessment of overall compositional similarity, a rapid MS-based "fingerprinting" technique that does not incorporate a chromatographic step is often used (Catchpole and others 2005; Fiehn and others 2005) prior to more rigorous analyses. Fingerprints such as flow injection electrospray ionization (FIE)-MS can be regarded as simplified images of total sample composition in that the measured variables (m/z) depend not only on the levels of a measured metabolite but also on the levels of coeluting material that can contribute to matrix effects or ion suppression. The low resolution adopted in fingerprinting approaches (typically 1 atomic mass unit) also implies that a single m/z value is compiled by integrating the levels of more than 1 metabolite (for example, members of a homologous series or isomers).

Multivariate fingerprint analyses can be used to highlight compositional similarities or dissimilarities. Where compositional differences between a novel crop and a conventional standard are preliminarily identified, a more rigorous comparative assessment process, using validated and quantitative compositional analysis methods, can then be pursued in order to determine whether additional safety assessments are needed.

Defining compositional equivalence is not easily achieved using standard statistical methods. Unsupervised data analysis techniques such as principal components analysis (PCA) look for regularities in unlabeled data. Supervised techniques such as

linear discriminant analysis (LDA) and decision tree analysis build models that discriminate between labeled data. However, for compositional equivalence, the interest is in data similarity rather than the ability to discriminate between classes. It has been suggested that if an unsupervised algorithm clusters metabolome samples close together, they can then objectively be considered as similar, and if classes cannot easily be discriminated by supervised methods, then they are, in effect, objectively similar (Catchpole and others 2005).

The comparator approach's strength has recently been demonstrated in a study in which field-grown tubers from conventional potato cultivars were compared to GM potatoes that contained high levels of inulin-type fructans (Catchpole and others 2005). Two classes of experimental GM potato lines developed in the Désirée cultivar were investigated. The 1st transgene coded for the enzyme sucrose:sucrose 1-fructosyltransferase (1-SST), which transfers a fructosyl residue from 1 sucrose molecule to another, producing the trisaccharide 1-kestose, and oligofructans with up to 5 degrees of polymerization (DP) (Hellwege and others 2000). The 2nd transgene was fructose:fructose 1-fructosyltransferase (1-FFT), the product of which utilizes 1-kestose (and other oligofructans) to build inulin polymers (Hellwege and others 2000). The acquired data highlighted large variation in the metabolic profile and composition of the conventional cultivars. In the context of the comparative safety assessment process, it demonstrated that the metabolite composition of field-grown inulin-producing potatoes was within the natural metabolite range of classical cultivars. The GM potatoes were, in fact, very similar to the progenitor line, Désirée, with the exception of the intentional elevation of fructans and their expected derivatives. In the comparative assessment framework, such metabolic side products would be subjected to follow-up assessment.

The cultivar-based compositional heterogeneity that has been described by Catchpole and others (2005) emphasizes the importance of comparison with a range of equivalent cultivars and not solely the parental line. For example, although the trisaccharide 1-kestose metabolite was not found in the genetic background line of the GM plants, a trisaccharide indistinguishable from 1-kestose was found in the other field grown conventional tubers. GC-TOF (time of flight) analysis highlighted similar findings and indicated that metabolic changes observed in conventional breeding approaches were, in these cases, comparable in magnitude to those resulting from modern biotechnology.

The value of targeted metabolic profiling has also been demonstrated in a recent evaluation of chromatographic methods for the highly selective detection and quantification of the 2 major potato glycoalkaloids, α-chaconine and α-solanine. High-throughput capabilities for > 1000 analytical runs were developed for electrospray-tandem mass spectrometry in multiple reaction monitoring mode coupled with either reversed-phase (RP) or hydrophilic interaction (HILIC) columns (Zywicki and others 2005). The RP method was more precise, more accurate, and more rugged under field conditions than the HILIC method for maintaining peak shape symmetry over 1000 analyses. In a study on the potato peels of 6 GM lines, the glycoalkaloid content in potato peels was found within the range of 6 conventional cultivars (Bushway and others 1983). This is consistent with the work of Catchpole and others (2005), described previously, who also found no differences between conventional and GM cultivars in these metabolites.

2.5.2 Proteomics

For more than 30 y, proteomics and protein profiling have been used as research tools to comprehensively analyze protein abundance and diversity. Just as there are metabolic compounds with known hazards, there are proteins known to cause allergenic or other toxic effects and, thus, might need to be monitored. In principle, monitoring proteins can be accomplished with selective and sensitive techniques such as western blots or "mass westerns," which employ LC and triple quadrupole MS for specific fragments and mass transitions of target proteins. Unfortunately, there is no prediction tool that allows an unbiased calculation of the likelihood that any given protein, or a specific protein posttranslational modification (for example, glycosylation, phosphorylation, or any of the other 200 known modifications), would raise safety concerns with respect to potential toxic or allergenic effects.

It has been suggested that nontargeted "fishing" for novel proteins could provide a preliminary indication of potential concern. For that information to be useful, however, validation and statistical data analysis strategies would need to achieve the same level of precision, repeatability, and so on as that required for targeted metabolite analyses that are currently used for safety and nutritional assessments of GM crops. First, the proteomic approaches must establish that they can be used in a robust and reliable manner, giving consistent and precise quantification results for a wide range of proteins. In principle, 2 methods can be applied for the nontargeted comparisons of relative protein abundances: either classical 2-D gel electrophoresis using colorimetric staining for detection and relative quantification, or chromatography-based separations followed by MS detection and quantification (LC/MS).

The 2-D gels can be used today at comparatively low costs and reasonably high precision; however, this technique usually does not detect integral membrane lipophilic proteins and proteins of either very high or very low masses and extreme isoelectric points (pI). In addition, spot detection, gel alignments, and automated quantification methods must be shown to work reliably for standard food and feed crops before proteomic databases can be set up to unambiguously determine when changes for a specific protein in a new crop fall outside the natural range for that protein in that species. Recently, Ruebelt and others published a series of papers (2006a, 2006b, 2006c) demonstrating, with 2-D gel proteomics of *Arabidopsis thaliana* seed samples, that the method itself can reproducibly detect and quantify spots, but that there was a wide range of protein abundances when 12 different *Arabidopsis* varieties were compared. In fact, the range covered the abundance range of GM *Arabidopsis* lines. A similarly thorough study on potato varieties and GM lines concluded that proteome dissimilarities between different individual cultivar lines were far larger than dissimilarities between a GM line and its progenitor conventional counterpart (Lehesranta and others 2005). In principle, 2-D gel proteomics may be used if efforts are made to ensure quality control and there is a high enough sample throughput to cope with the statistical challenges.

A 2nd proteomic approach is to utilize LC/tandem MS (LC/MS) on fractionated or partly fractionated proteome samples (for example, by using online cation exchange fractionation steps before LC/MS separation of trypsin-digested proteomes). Such approaches can be used with or without labeling with stable isotope intermediates that are often employed to enhance the precision of quantification results. No reports have yet been published on plant proteomes with the same diligence toward precision and range assessments using this method as have been reported with the 2-D gel approach. In principle, proteome fractionation with subsequent LC/MS quantifications may work, but is still plagued by some fundamental challenges called ion suppression and retention time alignments. For either of the 2 general approaches on proteome quantifications, laboratories have to establish that they can perform protein quantifications in a high-throughput manner using quality control charts and precision analyses as minimum quality checks to be able to populate range assessments of crop proteome databases.

2.5.3 Transcriptomics

Several laboratories investigated the potential value of transcript profiling for the safety assessment of GM food plants (Kuiper and others 2003; Baudo and others 2006). A study of tomatoes showed that different stages of ripening could be identified based on reproducible differences in gene expression patterns (Kuiper and others 2003). Detailed global gene expression profiles with a series of GM and conventionally bred wheat lines showed that differences in endosperm and leaf transcriptome profiles between conventional and related GM lines were consistently extremely small (Baudo and others 2006). Differences in gene expression in the endosperm among conventionally bred materials were much greater than differences between GM and conventional lines exhibiting the same complements of gluten subunits (Baudo and others 2006). These results suggest that the presence of the transgenes did not significantly alter gene expression. However, for transcriptomics to be useful, the variability for each transcript needs to be established and knowledge gained on the relevance of each new assay point regarding safety and nutrition.

2.5.4 Conclusions and recommendations about profiling

Nontargeted methods have been established for metabolite, protein, and transcript analyses. Although these profiling techniques may prove useful to identify differences among tissues or between a food component from a GM product and its conventional counterpart, the relevance to safety assessment has yet to be established. Hence, these methods may be useful during a product's developmental phase because they can help focus the safety assessment process by identifying the specific compounds that may need to be measured in a nutritionally improved product. It remains important to monitor novel foods and feeds by targeted metabolite analyses for nutritional value and antinutrient and toxicant levels to detect alterations in the composition of the nutritionally improved crop as part of a nutritional and safety assessment of the GM crop. Targeted approaches for the detection of specific differences between novel plant lines and a variety of comparator lines must adequately address natural variability and quality control measures. In addition to range levels that are recommended for these compounds based on health considerations, it is recommended that profiling databases for crops are established that span wide environmental conditions. Such databases will enable baseline assessments to which profiles of metabolites, proteins, and transcripts in novel foods may be compared, whether they are generated using molecular biology techniques or otherwise. At present, the nontargeted approaches complement existing direct approaches for detecting specific differences. For nontargeted profiling methods to be useful for nutritional and safety assessment purposes, the methods must be validated and harmonized globally.

2.6 Safety of Animal-Derived Foods (Meat, Milk, and Eggs)

At the time of the ILSI (2004) publication, it was concluded that one could assume nutritional equivalence of GM crops with agronomic input traits if compositional equivalence was established and that longer-term livestock feeding studies added little to the nutritional assessment (Flachowsky and others 2001).

However, a number of hypothetical safety concerns continue to be raised, including the potential for introduced DNA and/or the protein encoded by the transgene to transfer to animal-derived products intended for human consumption. This concern has been addressed by the FAO, WHO, FDA, and the OECD (FAO/WHO 1991; FDA 1992; OECD 2003b). These organizations stated that the consumption of DNA from all sources—including GM plants—is safe and does not produce a risk to human health, given the long history of safe consumption of DNA. Nevertheless, the search for fragments of transgenic DNA in the digestive tract of livestock and their presence in foods such as milk, meat, and eggs continues.

It has been noted that although DNA fragments from endogenous multicopy genes, such as *rubisco*, have been detected in animal tissues, fragments of transgenic DNA have not been widely detected in animal-derived products (Flachowsky and others 2005; CAST 2006). Thus, the relative frequency of a gene in the plant affects the probability of it being found in an animal tissue—DNA from endogenous multicopy genes is far more abundant than that of the transgene.

A recent review reported on the detection of DNA in animal-derived products in 15 feeding studies conducted since 2003 (Flachowsky and others 2005). These studies were conducted in a range of livestock species, and although fragments of DNA from multicopy endogenous genes were found in some tissues, no fragments of transgenic DNA were detected in any animal-derived products. These findings are supported by a recent and detailed study with poultry (Deaville and Maddison 2005). In this poultry study, although 23% of all animal samples contained fragments of the multicopy *rubisco* gene fragments, fragments of transgenic DNA were not detected in any animal tissues, even though fragments of the transgene were still detectable in feed samples traversing the upper gastrointestinal tract.

In 2006, a study using an assay with greatly enhanced analytical sensitivity identified very small fragments (106 to 146 base pairs [bp]) of the transgenes *cry1Ab* and *cp4 epsps* in conventional and "organic" milk samples in Italy (Agodi and others 2006). "Organic" milk is supposed to come from animals not fed GM crops, so the source of the fragments of these transgenes is debatable. In addition, the fragments of transgenic DNA

reported in milk were so small that the results must be interpreted in the context of the minimal size for a functional gene. For example, the size of the intact *cry1Ab* gene is 3500 bp, and its minimal functional unit is encoded on 1800 bp of DNA, both of which are considerably larger than the detected 106 to 146 bp DNA fragments.

To conclude, therefore, the detection of DNA in milk, meat, and eggs is likely to be a function of the abundance of the gene, the size of the DNA fragment being tested, and the sensitivity of the analytical methods, such that detecting fragments of transgenic DNA would be much rarer and more difficult than detecting high copy number endogenous genes. Given the long history of safe consumption of meat, milk, and eggs from animals in which plant gene fragments have now been shown to be detectable, and given that the DNA of a transgene is identical to all other types of DNA, then products from animals fed GM crops would not differ from foods already deemed safe (Jonas and others 2001).

2.7 Application of Risk–Benefit Analysis to Improved Nutrition Crops

In the Codex Alimentarius model, risk analysis is composed of 3 elements, risk assessment, risk management, and risk communication (Figure 2-1). The aim is to ensure the highest appropriate level of public health protection, as well as to strive for transparency of risk management and continuous communication between assessors and managers during the process. During risk analysis, the risks have to be weighed against other issues, such as the expected impacts, which require an understanding of these issues within the context of the levels of health protection that are considered appropriate. For nutritionally enhanced crops, it is particularly important to balance the intended positive health impact such as a significant improvement in health and a significant decrease in disease, suffering, and/or death against the outcome of the risk characterization. The impact of nutritionally enhanced crops may alternatively be viewed as a reduction in harm caused by control of a preexisting risk such as malnutrition. The case studies in this document focus on recommended scientific assessments of possible risks associated with new nutritionally improved food or feed.

The issue that science-based risk assessments, as currently performed, do not balance the potential risks against the intended positive nutritional impact (Boobis 2006) was discussed with experts during a workshop in preparation of this document (ILSI 2006). The workshop participants recommended that the risk assessment of improved nutrition foods and feeds follow current internationally harmonized food safety assessment procedures and be completed regardless of the potential positive impact of the improved nutrition crop. It was also recommended by the workshop participants that, similar to current risk assessment practices, if the positive nutritional impacts are to be assessed, the methodologies must be similarly detailed and science-based and should account for regional variation in factors such as processing, consumption, and health issues.

It is likely that different groups of scientists are involved in assessing risks from those assessing positive impacts. Those assessing positive impacts may be instigated by the need to verify claims of a positive health effect of a particular food or feed. Assessment of the positive nutritional impact of a novel food in the target population(s) must also consider its projected use and intake once it is commercially available. The workshop participants also noted that some nonscientific issues may be linked with the assessment of potential positive impacts of improved nutrition foods that are not within the domain of scientific risk assessors, but may be appropriate for consideration by risk managers (Figure 2-2).

Figure 2-1—Risk analysis model (adapted from FAO/WHO 1997).

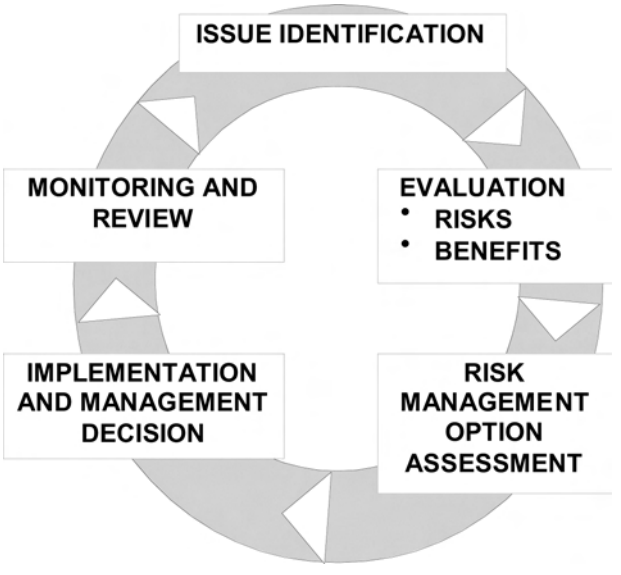

Figure 2-2—Risk management process (adapted from FAO/WHO 1997).

Some scientists have begun asking if the premarket safety assessment used in many countries is attempting to achieve a standard of absolute safety by continually adding data requirements as newer analytical methods come available (Bradford 2005a, 2005b). These scientists suggest that, from a scientific perspective, the cumulative experience over 2 decades of assessing GM crop safety should allow us to determine which tests need to be applied to new GM varieties to determine if they are as safe as their traditional counterparts with a history of safe consumption. This process is relatively simple for some crop species, while others may require more extensive analysis—yet today all crops species undergo the same assessment process, regardless of risk. The fundamental concept of the comparative risk assessment process is that it provides a reasonable degree of certainty that a new GM variety of a crop is as safe as the conventional varieties currently being safely consumed by humans and animals.

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Chapter 3: Double-Embryo, High-Protein, High-Oil Maize Produced Using a Cytokinin-Based Flower Rescue

ABSTRACT: Maize is an important staple of human diets and animal feed. Improving the nutritional profile for both of these purposes is a desirable research focus and agricultural endpoint. Many mechanisms have been used to improve maize protein and oil content, including selective breeding to introgress desirable traits (Quality Protein Maize), introducing single natural or synthetic genes expressing desired traits, changing signal sequences (high methionine maize), and modifying metabolic pathways (high-lysine maize, see Chapter 6). A novel approach was used to indirectly increase protein and oil content (Young and others 2004). Maize produces unisexual florets. Within the maize spikelet, the meristem gives rise to an upper and lower floret and male (tassel)- and female (ear)-specific florets are borne on separate inflorescences. The lower floret of each ear spikelet is aborted early in its development, leaving the upper floret to mature as the only female floret. Expression of a bacterial cytokinin-synthesizing isopentenyl transferase (IPT) enzyme, under the control of the *Arabidopsis* senescence-inducible promoter SAG 12 (senescence associated gene), blocked the abortion of the lower floret and resulted in 2 functional florets per spikelet. The pistil in each floret was fertile, but the spikelet produced just 1 kernel composed of a fused endosperm with 2 viable embryos. The 2 embryos were genetically distinct, indicating that they had arisen from independent fertilization events. The embryo contains most of the protein and oil in the kernel and kernels that contained 2 embryos have more protein than conventional maize. The presence of 2 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 and others 2004).

3.1 Demonstrated Trait Need

Cereal grains are the most important crops for humanity, representing the staple diet for two-thirds of the world's population (FAOSTAT 2004). Beyond that, they are a principal staple for animal feed and for production of valuable commodities such as oil, protein, and starch. Today, there is great variation in nutritional composition of many maize varieties (Table 3-1). Maize, the most widely produced feed grain in the United States, accounts for more than 90% of the total production of feed grains in the country, with > 32 million hectares of land planted with maize. In the United States, the majority of maize (> 50%) is used to feed animals for meat, milk, and egg production (NCGA 2007). Much of the remainder is exported for feeding animals or made into maize sweeteners or fuel alcohol.

While maize may not comprise a significant component of the human diet in North America, as described in more detail in Appendix 2, protein-energy malnutrition (PEM) is the most lethal form of malnutrition and affects every 4th child worldwide according to the WHO (2006). The FAO estimates that 850 million people worldwide suffer from undernutrition, of which insufficient protein in the diet is a significant contributing factor (FAO 2005). For animal diets, there is interest in maize oil; as noted, approximately 70% of all maize grown in the United States is fed to domestic livestock in the form of ground grain (including high-oil and high-moisture maize) and maize silage.

Researchers have tried for some time to breed high-quality protein maize with some success. One type, called Quality Protein Maize is a natural mutant, known as *opaque2 (o2)*. Weanling rats fed a diet of 90% *o2* maize gained weight more than 3 times faster than those fed a diet with standard hybrid maize (Maffia and others 1976). In a number of regional studies, including Guatemala, Peru, Colombia, and India, various parameters were measured such as apparent nitrogen retention and biological value. For children, *o2* maize was generally found to be nearly equivalent to milk protein in meeting protein nutritional requirements and in aiding recovery from kwashiorkor, a protein deficiency disease (Nelson 2001). However, breeding for trait improvement has limitations that include significant reduction in yield, slow drying kernels that are more subject to fungal infections, soft endosperm that easily breaks under typical harvesting and milling practices, and lysine content that was unusually susceptible to environmental fluctuations, complicating efficient diet formulation.

The most extensive breeding program for developing high-lysine maize based on the *o2* mutant, but with more acceptable agronomic and grain handling characteristics, was conducted by the Intl. Maize and Wheat Improvement Center in Mexico (Centro Intl. de Mejoramiento de Maiz y Trigo, CIMMYT) and resulted in Quality Protein Maize. The center introduced modifiers that increased endosperm hardness to a more desirable phenotype, and there was continued selection for yield and other performance aspects. This variety is now being grown in regions such as Africa and China, but requires continual breeding to ensure that the mutation is maintained in the crop.

Unlike many less-developed regions in the world, only a fraction of maize grown in the United States is used for human food. Up to 70% is used to feed livestock, 20% to make the maize starches and oils that are key components of many foods, and approximately 5% is milled for dry products such as maize chips. Therefore, in a field in which increases in oil, protein, or starch content as small as 1% are considered a significant achievement, any technology that provides the ability to significantly increase any one of these would be viewed as a considerable advancement. Scientists at the Univ. of California, Riverside have used a

peculiarity of certain plants that has evolved as a reproductive strategy to achieve such an endpoint for two of the principal components in maize, protein and oil content.

3.2 Experimental Procedure

To put the current work in context from a nutritional and safety assessment perspective, it is important to provide some background on kernel development in maize. Unlike the majority of plant species, maize produces unisexual florets that have only either male (stamen) or female (pistil) parts, but both male and female flowers appear on the same plant (monoecious). For monoecious species, the sex determination process by which sexual organs form in each floret is rather complex. Within the maize spikelet, the meristem gives rise to an upper and a lower floret, and male (tassel)- and female (ear)-specific florets are borne on separate inflorescences. The male stamen (tassel) is responsible for producing pollen. The female pistil (ear) bears the ovaries that give rise to kernels after pollination. Although initially bisexual, floret unisexuality is established through selective organ elimination and abortion of specific floret organs. Female initials are eliminated from florets in the tassel inflorescence, resulting in male florets; conversely, male initials are eliminated from florets in the ear inflorescence, resulting in female florets. In addition, the lower floret of each ear spikelet is aborted early in its development, leaving the upper floret to mature as the only female floret. Arrest of pistil development in the lower floret is followed by degeneration, which initiates in the pistil primordium (precursor cells).

As with many organisms, this selective loss is achieved through apoptosis or programmed cell death (PCD), a fundamental process that cuts across the boundaries of many scientific disciplines. In the context of maize reproduction, it is interesting that the Greek term apoptosis traces its roots not to a medical phenomenon, but rather to the process of a petal falling from a flower or the loss of a leaf from a tree. As with other apoptotic applications in plant development, the process results in discarding tissues or organs that are no longer required, modifying existing organs, or adapting to environmental changes. Induction of PCD is a fine balance between the withdrawal of positive signals; that is, signals needed for continued survival, and the receipt of negative signals. The continued survival of most cells requires that they receive continuous stimulation from other cells.

There are several naturally occurring maize mutations called *tasselseed* (for example, *ts1 and ts2*) that result in female flowers developing on the male tassel. The *ts2* mutation has been cloned and encodes a short-chain alcohol dehydrogenase. Normal expression of *ts2* in the pistil primordium of male flowers induces apoptosis and leads to pistil abortion and male tassel development. Both genes are suppressed by *silkless1* (*sk1*). Loss of *sk1* leads to the abortion of both upper and lower floret pistil in ear spikelets. In contrast, loss of *ts1* or *ts2* function results in the sexual maturation of pistils in all florets, tassels, and upper and lower florets of each ear spikelet, leading to a complete female takeover. Expression of *ts2* seems to switch on PCD because it coincides with the loss of nuclei in the pistil primordium. The presence of *Sk1* basically eliminates *ts2* activity in the upper floret pistil by blocking PCD, although *ts2* is expressed in all pistil primordial cells (Calderon-Urrea and Dellaporta 1999). Mutations at 2 other loci (*pi1/pi2*) differentially suppress pistil abortion in the lower ear floret, but not in the tassel spikelets (Veit and others 1993). The fact that this demonstrates a mechanism (even if the actual process is unknown) whereby one can rescue the lower floret pistil, while not affecting pistil abortion in tassel spikelets (thus not affecting male fertility), makes for a potential intervention point that was taken advantage of with some unexpected payoffs from a nutritional perspective (Young and others 2004). Intervention was achieved by the judicious use of plant growth regulators (PGRs) rather than direct manipulation of gene activity, per se.

It had already been demonstrated in tobacco leaves that cytokinin plays a key role in regulating entry into a senescence program. Cytokinins are adenine-derived compounds that promote cell division and have other similar functions to kinetin. Gan and Amasino (1995) developed a system based on cytokinins to study leaf senescence because it has important agricultural implications. For example, senescence may limit yield in certain crops, and it also contributes to the postharvest loss of vegetable crops. Therefore, studying leaf senescence could lead to ways of manipulating senescence for agricultural applications. Gan and Amasino (1995) developed a construct to study the effect of cytokinin by placing the *Agrobacterium* cytokinin-synthesizing IPT gene under the control of the *Arabidopsis* senescence-inducible promoter from the cysteine

Table 3-1—Nutritional values of corn grain.

	Nutritional values of corn grain (wt per 100 g)															
	Moisture (g)	Energy (kcal)	Protein (g)	Fat (g)	CHO (g)	Dietary Fiber (g)	Ash (g)	Ca (mg)	P (mg)	Fe (mg)	B1 (mg)	B2 (mg)	B6 (mg)	Niacin (mg)	Lys (g)	Trp (g)
Dent	14	343	7.5	3.6	70.3	8.1	1.3	15	290	3	38	14	53	280	2.3	0.5
QPM	14	350	9	3.8	69.9		1.3	9	232						3.7	0.9
Sweet	76.9	81	2.6	1	18.6	0.2		9		0.8	0.03	0.01		0.7		

Data extracted from Dickerson (2003), Saldana and Brown (1984), USDA (1976–1993), and Valverde and others (1981), no data available in empty cells.

protease gene, SAG, which induces senescence. There are about 30 different SAG-coding degradative enzymes, such as RNases, proteinases, and lipases, and genes with products involved in nutrient translocation processes. Interestingly, the group also includes genes homologous to short-chain alcohol dehydrogenases similar to *Ts2*, which is a member of the short-chain alcohol dehydrogenase family. As also noted, *Ts2* is required for PCD during sex determination in maize (DeLong and others 1993), and the existence of 2 members of this class of genes in different PCD programs is intriguing.

The PGRs regulating senescence and PCD can be similar, although these processes are distinct developmentally. The former typically occurs as the last developmental stage of an organ, whereas the latter can initiate even at early developmental stages. For instance, ethylene, a simple gaseous hydrocarbon PGR, promotes the senescence of leaves as well as the PCD of root cortical cells in response to hypoxia (Grbic and Bleecker 1995; He and others 1996). To investigate whether cytokinin may affect the abortion of maize floral organs, a construct, similar to that used by Gan and Amasino (1995) with the cytokinin-synthesizing IPT enzyme under the control of the *Arabidopsis* SAG12 (senescence-inducible promoter), was introduced into maize (Young and others 2004). The selectable marker used was bialophos resistance. The hypothesis that expression of IPT would be observed in the ear during early floral development was confirmed by experimentation. Abortion of the lower floret pistil in the ear was suppressed, resulting in a spikelet with 2 florets that each contained a fertile pistil. Neither the number nor arrangement of florets within a spikelet, nor organ number within each floret, was altered, demonstrating that IPT expression did not alter either spikelet or floret determinacy. Somewhat surprisingly, the pistil in each floret was fertile and pollination resulted in the production of a single kernel from each ear spikelet composed of a fused endosperm with 2 viable, normal size, genetically distinct embryos, indicating that fusion between the 2 pistils within a spikelet had occurred. Because the 2 embryos were genetically distinct, this indicates that they had arisen from independent fertilization events.

These results suggest that cytokinin can determine pistil cell fate during maize floret development. The embryo contains most of the protein and oil in the kernel, and kernels that contained 2 embryos have more protein than normal maize. The presence of 2 embryos in a normal-sized kernel meant that something had to be reduced, leading to displacement of endosperm growth. This resulted in kernels with an increased ratio of embryo to endosperm content. The end result is maize grain with more protein and less carbohydrate (personal communication, Daniel Gallie 2005, unpublished data).

3.3 Considerations for Safety and Nutritional Assessment

The majority of products on the market to date have been modified for agronomic "input" traits. Although the use of a PGR induction system may at first seem to differ substantially from the current commercialized applications, the same rigorous assessment system in place for current assessments should suffice to address all nutritional and safety concerns. A meticulous safety testing paradigm using a systematic, stepwise, analytical, and integrated safety assessment approach has been developed and implemented for GM crops. The resultant science-based process focuses on a classical evaluation of the toxic potential of the introduced novel trait and the wholesomeness of the GM crop. In addition, detailed consideration is given to the history and safe use of the parent crop, as well as that of the gene donor(s). There are some specific assessments of double-embryo maize that are appropriate for consideration in determining food and feed nutritional and safety appraisal.

3.3.1 Agronomic aspects

Safety issues aside, it will take many years to determine whether double-embryo maize is suitable for commercial production. One of the key issues is that each kernel will produce 2 plants because each contains 2 embryos. Two seedlings growing from the same kernel or seed is inefficient because they compete for nutrients and resources. Determining how well these plants grow and perform when they are competing for resources will be an important field test. From an economic perspective, if farmers are to adopt a new variety, it must perform at least as well as today's commercially grown maize varieties. Possibly, this trait could be bred into other elite strains of maize and selected against different genetic backgrounds.

In addition, setting aside safety and nutritional considerations, because distribution for this trait is expected to be global, especially in less developed countries, researchers must consider that the high levels of fat and protein do not interfere with people's ability to mill and cook the grain. If modified cultivation, processing, or preparation is required, then this may affect adoption.

3.3.2 Nutritional safety issues

The nutritional endpoint of higher protein and oil and lower starch presents no known nutritional limitations or safety implications for these targeted endpoints. No change is anticipated from present expected dietary impact and consumption levels for maize. An increase in cytokinin-synthesis, which is part of a well-understood pathway in maize and involves specific cells undergoing programmed cell death, is not expected to have any negative nutritional or safety concerns. This is especially true considering that once sufficient cytokinin is produced to retard senescence (or in this instance, apoptosis), the activity of the senescence-specific promoter is attenuated.

R.E.D. FACTS (1995) reports that the plant growth regulator cytokinin was initially registered in the United States in 1978 as CYTEX (EPA Reg. NR 35980-1) for application to certain citrus, fruit, and vegetable crops. During Phase Four of the accelerated pesticide re-registration process, the database for cytokinin was evaluated and determined to be inadequate in satisfying certain requirements for biochemical pesticides that include plant regulators. A data call in (DCI) was issued by the U.S. Environmental Protection Agency (U.S. EPA) in August 1993 to fill the outstanding data gaps. Since the DCI, the initial EPA position regarding these data gaps was reevaluated. All of these data requirements, except a nontarget insects study, were waived because available information indicated that cytokinin does not cause unreasonable adverse effects in that (1) the principal sources of cytokinin, algae and seaweed, are natural components of fish diets, (2) cytokinin has minimal acute mammalian toxicity, (3) cytokinin is used as a dietary supplement in animal

feeds, and (4) cytokinin pesticide products are expected to have no adverse effects to fish and wildlife. The report (R.E.D. FACTS 1995) further states that the acute dermal toxicity (LD > 2 g/kg), eye irritation (slight irritation), and dermal irritation 50 (slightly irritating) place cytokinin in Toxicity Category III (the 2nd lowest of 4 toxicity categories). Cytokinin's oral toxicity 50 (LD > 5 g/kg) places it in Toxicity Category IV (the lowest of 4 toxicity categories[1]).

In 1998, the U.S. EPA proposed a growth regulator tolerance exemption regulation (including cytokinin) to facilitate adding new crops, application rates, and uses to the labels of products containing the listed active ingredients when used as plant regulators (EPA 1998). The U.S. EPA determined that tolerance exemptions for these types of substances are usually based on the results of subchronic feeding, developmental toxicity, and mutagenicity studies. However, for many of the plant regulators, some or all of these study requirements have been waived because of negligible exposure from very low use rates. Such use rates for these active ingredients are expected to be effective when these substances are used as plant regulators, and these low use rates are not expected to significantly increase dietary intake over that anticipated from consumption of a normal diet because the subject active ingredients are naturally occurring (or are synthesized to approximate the naturally occurring forms) in plants. The U.S. EPA observed, parenthetically, that plants are part of a normal human diet and all plants produce cytokinin.

The U.S. EPA noted that these substances are effective plant regulators when applied at low rates, but are often herbicidal when applied at high rates. The toxicological data they presented demonstrate that testing at high doses yields few effects in laboratory animals. Doses high enough to cause toxicity in animal studies would represent application rates toxic to crops (high, herbicidal rates). The EPA specifically noted that no additional toxicity data are needed for cytokinins because they are naturally occurring in numerous plant food sources and are available as a food supplement. In studies using laboratory animals, cytokinin has generally been shown to be of low acute toxicity. These evaluations all refer to topical applications of cytokinin, so its increased expression within the plant tissue should be assessed.

As measured so far, the SAG promoter expression levels will be low and should only occur in specific cells undergoing PCD. The metabolism of the target nutrients, protein and oil, within humans and animals is very well understood. From studies to date, there are no expected or unexpected pleiotropic changes in nutrients and antinutrients or intermediary metabolites in the maize (personal communication, Daniel Gallie 2005, unpublished data). Also, there have not been any adverse effects on expression of other plant genes directly or indirectly through transcription factors or mRNA stability.

3.4 Genetic Modification Details

3.4.1 Inserts

Embryogenetic maize callus from the HiII inbred line (derived from A188 B73) was used for transformation by particle bombardment. One hundred sixty-eight plants were regenerated from 18 transformation events as described by Armstrong (1994). Transgene-containing plants were crossed to B73, and the progeny were selfed. Rescue of the lower floret was examined in T3 kernels of self-pollinated T2 plants.

3.4.2 Selectable marker: *bar* gene

Plasmid pAHC20 (Christensen and Quail 1996) contains the selectable *bar* gene (phosphinothricin acetyl transferase) that confers tolerance to the herbicide Basta (active ingredient glufosinate ammonium) under the control of the maize ubiquitin promoter. This plasmid was codelivered with the *SAG12*-IPT construct to select for transformants on the plant selective antibiotic, bialaphos (the enzyme phosphinothricin acetyltransferase inactivates phosphinothricin, the active ingredient of the herbicide bialaphos, by acetylation). The biochemical and toxicological characteristics of glufosinate have made it a popular, nonselective herbicide, which has been commercialized as Basta® and Liberty® by Bayer CropScience. Some microorganisms can detoxify glufosinate by producing an enzyme that catalyzes acetylation of the amino group. The *bar* and *pat* genes, isolated from different *Streptomyces* species, both encode a phosphinothricin acetyltransferase (PAT) and are widely applied in plant genetic engineering. The gene from S. *hygroscopicus* has been referred to as *bar* (for bialaphos resistance) and that from S. *viridochromogenes* as *pat*. Treatment of genetically modified plant cells carrying a *bar* gene with glufosinate or bialaphos provides a very efficient means of selection in transformation protocols. Although the double-embryo maize will be marketed for the intended increase in protein and oil, the presence and expression of the *bar* gene enable tolerance to commercial applications of the herbicide glufosinate ammonium and, therefore, this product should also be regarded as a herbicide-tolerant crop.

3.4.3 Active construct

Achieving floret rescue using cytokinin required a system that avoided undesirable pleiotropic effects. In the cytokinin biosynthesis pathway, the 1st committed and controlling step is catalyzed by IPT. Therefore, expression of this enzyme will result in the production of cytokinins. The native plant IPT has not been identified, but a bacterial version (*Agrobacterium*) is available (*Agrobacterium* infection can cause a cytokinin-induced tumor to develop). However, having an expressed gene is not sufficient in and of itself. Cytokinins regulate a variety of developmental processes in addition to leaf senescence, such as rooting and apical growth (McKenzie and others 1998); cell division and assimilate import and partitioning (Brenner and Cheikh 1995); and vascular tissue differentiation (Aloni 1995). To avoid direct interference with these other aspects of normal plant development, Gan and Amasino (1995) cloned a highly senescence-specific SAG12 promoter to target cytokinin biosynthesis to the senescing tissues in transgenic tobacco plants. Transgenic tobacco plants expressing this chimeric gene do not exhibit the developmental abnormalities usually associated with IPT overexpression

[1]For acute oral, dietary, mammalian/avian/aquatic toxicity: Category I = very highly or highly toxic, Category II = moderately toxic, Category III = slightly toxic, Category IV = practically nontoxic.

because the system is targeted to senescing leaves and negatively autoregulated, thus preventing developmental abnormalities. Because sufficient cytokinin is produced to retard senescence, the activity of the senescence-specific promoter is attenuated. Senescence-retarded leaves exhibit a prolonged, photosynthetically active life span. This result demonstrates that endogenously produced cytokinin can regulate senescence and provides a system to specifically manipulate the senescence program without further phenotypic developmental abnormalities (Gan and Amasino 1997). Taking this into consideration, the construct used is p*SAG12*–IPT, composed of the *Agrobacterium* IPT gene under the senescence-specific *Arabidopsis* SAG12 promoter (Gan and Amasino 1995). In this instance, the construct targets the female pistils undergoing apoptosis (as opposed to senescence) and rescues them, resulting in a fertile floret.

3.4.4 Proof of gene insert in transgenic plants

The presence of the IPT transgene was determined by polymerase chain reaction (PCR) in seedling leaves using HotStarTaq (Qiagen) in a reaction containing dNTPs (deoxynucleotide 5′-triphosphate), MgCl2, forward primer (SAG12F2; CGTACGTATCCCTCTTGTCGTCTAATGA), and reverse primer (IPTR1; CGTTCCTTTCAGTTCTTCCACTGTTGGT). The principal basic concern is unexpected, unintended pleiotropic effects of adverse cytokinin expression. However, given the specificity of the SAG12 promoter, this is unlikely to occur and if it does, such events should be easily eliminated early in the screening process because of growth and morphological abnormalities and, later, yield drag. However, these concerns center on commercialization rather than safety.

3.5 Recommended Future Analysis

3.5.1 Molecular analysis

An appropriate analysis should include characterization of the number of insertion sites, number of gene copies, and the presence/absence of fragments.

3.5.2 Comparative safety assessment analysis

The product development process that selects a single commercial product from hundreds to thousands of initial transformation events eliminates the vast majority of events that experience unintended changes caused by the inappropriate insertion (or expression) of the transgene construct, or the action of its resulting phenotype on unintended targets. In addition, the selected commercial product candidate event undergoes detailed phenotypic, agronomic, morphological, and compositional analyses to further screen for unintended effects that would limit commercial acceptance or product safety.

However, it is recommended that, as part of the safety assessment of the lead double-embryo maize line, compositional and nutritional analysis should be performed, using the parental line and other elite maize hybrids as comparators. OECD (2002) explains that such a comparison should include macro- and micronutrients, antinutrients, and natural toxicants, and should identify the similarities and differences between the double-embryo maize and the conventional counterpart. In addition, if this is to be adopted to meet nutritional needs, then while not specifically relevant to safety and nutrition assessment, agronomic performance and phenotypic characteristics of double-embryo maize should be compared to parental and other conventional maize hybrids to identify unexpected differences that might have resulted from introduction of the trait. A normal phenotype is an indicator that there have not been enough alterations to affect safety.

Based on the differences identified, further investigations may be carried out to assess the safety and/or efficacy of these differences on double-embryo maize. Some of the already known differences between double-embryo maize and conventional maize are the introduced protein and associated metabolites, including the changes to the cytokinin PGR levels. Besides the intended effects of the genetic modification, interactions of the inserted DNA sequence (and the products thereof) with the plant genome are also possible sources of unintended effects that are assessed as part of this comparative process. Feeding studies with rats and broiler chickens might also be done to determine the nutritional feeding value of the increased protein and oil in double-embryo maize.

The introduced IPT protein is not similar to any known allergens and is not derived from any allergenic protein families; therefore, it is unlikely to provoke an allergic response. While the digestibility of the IPT protein has not yet been determined due to its relatively small size (37.2 KDa) and nonextensive post-translational modification, there is a limited likelihood that it will be allergenic. Similarly, although acute toxicity studies with IPT have not been reported to date, the enzyme has no relationship to known toxicants and the protein as noted is likely to be present at very low concentrations in maize. If the protein is readily digested in simulated gastric fluid, then it is likely that acute oral toxicity studies would not add materially to the overall safety assessment.

3.6 Recommendations

3.6.1 Safety assessments

Recommendation 1. Foods and feeds from double-embryo corn should be evaluated for the potential impact on health, with particular focus on those components and associated metabolites specifically altered in the crop, when they are characterized.

Recommendation 2. Human dietary intake data and dietary intake forecast models should be developed for at-risk populations.

3.6.2 Nutrition assessments

Recommendation 1. Premarket assessments should be conducted to evaluate the potential impact on nutrient intakes of consumers, paying particular attention to the expected benefits obtained from higher protein and oil.

Recommendation 2. While feeding studies in laboratory animals and livestock species are unlikely to contribute to the detection of unintended effects in a new crop because they lack adequate sensitivity, in the case of double-embryo corn,

livestock feeding studies with target species should be conducted on a case-by-case basis to establish the nutritional benefits that might be expected.

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Chapter 4: Nutritionally Improved Sweetpotato

ABSTRACT: Sweetpotato is grown in many developing countries, and varieties can be white-, yellow-, orange-, red-, or purple-fleshed. This crop is a secondary staple food crop in parts of Eastern and Southern Africa, and an important component of animal feed in countries such as China. This case study describes and compares 2 nutritional improvements of sweetpotato. One improvement involves selecting and breeding orange-fleshed sweetpotato as a biofortified crop to reduce vitamin A deficiency in Africa. The 2nd improvement aims to increase both the quality and quantity of protein in sweetpotato through the introduction of the synthetic *asp-1* gene (Kim and others 1992; Prakash and others 1997). Nutritional issues considered include the role of the sweetpotato in human nutrition, with a focus on Africa, and its potential to combat vitamin A deficiency and undernutrition; and its role in animal nutrition, specifically through increasing both the level and quality of protein. The protein case study concentrates on the event TA3 developed at Tuskegee Univ. (Egnin and Prakash 1995, 1997), which has been shown to have no negative agronomic characteristics. In terms of safety, the history of sweetpotato use and the measurement of a number of antinutrient compounds in this crop, such as oxalic acid, trypsin inhibitor, and furanoterpenoid compounds, are considered. If the orange-fleshed sweetpotato are to be used in animal feed in a way not previously done, it is recommended that additional nutritional testing, such as for performance and bioavailability, be carried out in domestic animals. Four studies are recommended for the ASP-1 sweetpotato. First, testing the safety of the genetic modification with the *asp-1* gene and derived ASP-1 protein. Second, carrying out supplementary compositional studies focused on, for example, appropriate antinutrients, such as oxalic acid, trypsin inhibitor, and others where appropriate. Third, documenting the phenotypic properties of the sweetpotato line and its comparator grown in representative production sites. Fourth, measuring the performance of animals fed ASP-1 sweetpotato compared with those fed conventional sweetpotato varieties. These studies could use a suitable animal model; an ILSI task force formulated guidelines for this type of study in a report titled *Best Practices for the Conduct of Animal Studies to Evaluate Genetically Modified Crops* (ILSI 2003). Data on protein bioefficacy in the enhanced protein sweetpotato are available from 1 hamster study.

4.1 Introduction

Sweetpotato is a root crop that is grown in many countries (Woolfe 1992). It is grown predominantly for human food in Africa, whereas it is produced primarily for animal feed in China (Box 4-1). Sweetpotato roots vary in color, with orange-fleshed types being particularly rich in β-carotene, the most important provitamin A carotenoid. Irrespective of their color, all sweetpotato are low in protein quantity and quality (Egnin and Prakash 1997; Prakash and others 1997). Some orange-fleshed varieties are found naturally, while others have been developed through conventional breeding. Sweetpotato varieties improved through modern biotechnology have also been developed (Woodward and others 1999; Wambugu 2001). In both cases, selected orange-fleshed varieties are being further developed through participatory breeding initiatives with local farmer communities to incorporate agronomic traits that farmers want.

Box 4-1—Sweetpotato
- Grown in many countries, particularly China, for animal feed: roots and vines.
- Human food, especially in Africa: roots consumed boiled, baked, or fried or as processed products such as starch, noodles, cakes, or juice.
- Rich in carbohydrate and low in protein.
- Different colored types, including white-, yellow-, orange-, red-, or purple-fleshed, which vary in the concentration of provitamin A carotenoids.

The orange-fleshed sweetpotato (Figure 4-1) is one of the biofortified crops the HarvestPlus program is developing as part of the global effort to control vitamin A deficiency (www.harvestplus.org; see Appendix 1 on VAD). The aim of HarvestPlus is to make available varieties that can contribute significantly to meeting the recommended daily intake of vitamin A for preschool children. A number of food science and nutrition studies have been carried out, showing that most of the provitamin A carotenoid is retained after simple processing and that consumption of these products can improve the vitamin A status of deficient children. Beta-carotene has not been reported to be toxic at intake levels observed for orange-fleshed sweetpotato.

The use of ASP-1 protein to increase the protein quality and quantity in sweetpotato has been achieved by introducing the *asp-1* gene coding for the ASP-1 protein through recombinant DNA techniques at Tuskegee Univ. (Egnin and Prakash 1997). The genetically modified sweetpotato has the potential to improve its contribution to both the protein quantity and quality in human and animal diets in areas of the world where it is grown indigenously.

Sweetpotato has a history of safe use in humans as it is widely consumed, especially in Eastern, Western, and Southern Africa. This document contains case studies for the provitamin A-enriched sweetpotato lines developed by the Intl. Potato Center "Centro Intl. de la Papa" (CIP) using conventional breeding, and the protein-enriched sweetpotato developed by Tuskegee Univ. via a transgenic approach. In both cases, the origin of the improved crop is reviewed and the possible impact on both safety and nutrition is assessed.

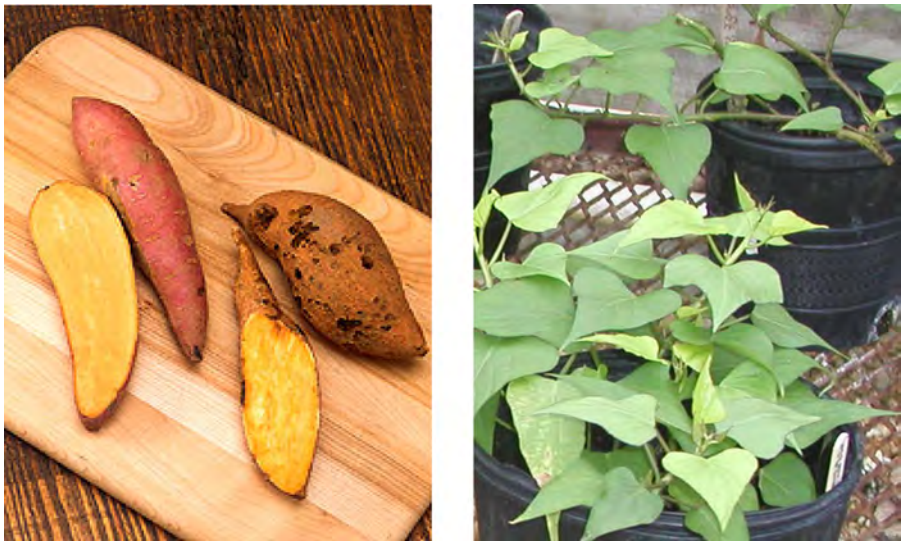

Figure 4-1—Orange-fleshed sweetpotato roots (left, photograph USDA-ARS/K9231–1/S. Bauer), and plants growing in pots (right, courtesy Tuskegee University, Ala., U.S.A.).

4.2 Sweetpotato and its Role in Nutrition

4.2.1 Origin and agricultural production

Sweetpotato (*Ipomoea batatas* L.) belongs to the morning glory family (*Convolvulaceae*), and both the storage roots and leaves are edible. It is thought to have originated in southern Central America. Early trading vessels disseminated sweetpotato throughout the world. Sweetpotato has long been an important food crop to many peoples, particularly to the poor, and is the 6th most important food crop in the world and 4th most important in the tropics (FAOSTAT 2004).[2]

Sweetpotato has a starchy and sweet taste, with different varieties having their own unique flavor profiles. They are often grouped into 2 categories depending on texture; some are firm, dry, and mealy when cooked, while others are soft and moist when cooked.

World annual production was > 127 million metric tons in 2004, 98.5% of which was in developing countries (FAOSTAT 2004). Asia accounts for the largest part of world sweetpotato production, which amounted to 112.4 million tons annual production in 2003 (FAOSTAT 2004). Production is greatest in China, accounting for 94% of the Asian sweetpotato production.

In contrast, African farmers produced about 11.4 million metric tons of sweetpotato in 2003 (FAOSTAT 2004). These data need to be interpreted with caution because of the difficulties in estimating production for a crop produced by small farmers on noncontiguous plots, harvested several times a year, and neither sold through regulated domestic marketing channels nor traded internationally in appreciable quantities. In many developing countries in Africa, Asia, Latin America, and the Caribbean, the sweetpotato is a secondary staple food or an important vegetable crop that is grown primarily by poor small farmers. Sweetpotato is typically a small farmer crop and often grown on marginal soils with limited yields. For example, in Kenya the average plot size is < 0.2 hectares, and about half of the harvest is kept for home use. Because sweetpotato is considered a "backyard crop," there are no reliable data on the extent to which it contributes to local food supplies. Overall, average yield in developing countries is low, at 15 metric tons per hectare, and there is wide variation. For example, African yields are about one-third that of Asian yields (CIP 2005). However, the sweetpotato is a reliable crop for small-scale production, and because it can be left in the ground for extended periods and harvested when needed, sweetpotato is especially appealing to small farmers (Qaim 1999).

[2]The book by Woolfe (1992) is an excellent source of general information on sweetpotato and has served as reference for many of the general considerations elaborated in the following sections.

4.2.2 Use of sweetpotato

Both the amount of sweetpotato consumed and the manner in which it is consumed vary widely. Average annual per capita supply of fresh roots for 2003 was estimated as Africa, 112 kg; Asia, 16 kg; Oceania, 18 kg; North and South America, 2 kg; Europe < 0.5 kg (FAOSTAT 2004). Sweetpotato consumption also varies within countries, by regions, by time of year, and by income group. Green tips are used as a vegetable in some areas and can be an important source of protein and micronutrients.

Sweetpotato is widely used by small farmers to sustain local livestock production systems. Almost everywhere that sweetpotato is cultivated, some part of the plant, in some form, is used in some type of animal production. Sweetpotato roots and vines are being increasingly used in pig and other livestock systems in China, and FAO estimated that 50 million tons in 2002 was used as feed (FAOSTAT 2004). About 45% of Asia's domestic sweetpotato supply is used for animal feed, and nearly 50% is used for human consumption, either as fresh or processed products (CIP 2005).

In contrast, 85% of Africa's domestic sweetpotato supply is for human consumption. For example, per capita sweetpotato consumption in Rwanda is estimated as 147 kg/y; Burundi, 120 kg/y; and Uganda, 88 kg/y, and most of this occurs in the drier seasons. Sweetpotato can be used as a seasonal staple when there is a shortage of other foodstuffs (CIP 2005). It is also an important crop in northern regions of South Africa. In Africa, commercial farmers use vines as fodder for livestock. Humans consume both the roots and young leaves and tips as green vegetables. Sweetpotato root is generally consumed boiled (and mashed) or

fried. It is also processed commercially or at home into flour that is then often mixed with other flours to make a composite flour. These flours are used to make porridge, which is an important complementary food for breastfed children. Less commonly, the flour is used to make bread and pastries. Poor households tend to eat boiled or baked sweetpotato roots. Fresh roots are also fried and the chips eaten as a snack food.

In China, humans mostly consume sweetpotato as a vegetable or health food, not as a staple food, and the roots are typically boiled or baked. Sweetpotato starch is used for making fresh or dry starch noodles and sheets, and for fresh starch cakes. Snacks of dry sweetpotato slices and bars are also popular. In Japan, consumers use many of the deeply colored root varieties and consume sweetpotato mostly as processed snacks, noodles, and candies. Sweetpotato is grown and the roots are consumed in the drier and poorer regions of India.

In Latin America, there is great diversity of varieties. Most sweetpotato roots are again eaten boiled or baked. Use varies in the Caribbean region. Sweetpotato is the main staple in some South Pacific islands, especially in Papua New Guinea highlands where many varieties are available, providing the dominant portion of the energy and protein requirements of the population (Okuda and others 1981; CIP 2006). In Oceania, in 2002, it was the most important tuber and root crop after potato (FAOSTAT 2004).

For long-term storage, the roots are sliced into thin chips for food or strips for animal feed that are sun- (usual) or shade-dried. Chips can be stored in the dried state, rehydrated, and then cooked. Alternatively, they can be ground into flour that can be mixed with wheat as a composite flour; the proportions of wheat and sweetpotato vary depending on taste. Pure or composite flour is used to make gruels, bread, donuts, cakes, and other products. Starch is extracted and used to make noodles, sheets, and cakes in China.

In some countries, processed products made from sweetpotato, including starch, noodles, candy, desserts, and flour, are made by farm households to extend the availability, diversify the use of, and increase the value of the crop. In China, in particular, production of sweetpotato starch in recent years has evolved into a cottage industry that uses millions of tons of roots per year as raw material inputs. The magnitude of these new uses is not easy to quantify in a systematic way; partly for that reason, the available statistics on processing do not always reflect their true level of importance (CIP 2005).

Sweetpotato is also consumed in Europe and the United States as a cooked vegetable. Sweetpotato has a low glycemic index, indicating low digestibility of the starch despite its high carbohydrate content.

4.2.3 Sweetpotato as source of vitamin A

Biofortification of sweetpotato through conventional breeding, which includes the selection of orange-fleshed varieties, is being done with the intent of controlling vitamin A deficiency in developing countries. This is because β-carotene is the most important provitamin A carotenoid and the predominant carotenoid found in orange-fleshed sweetpotato, and is more bioavailable than that in carrot or green leafy vegetables for instance (for example, van Jaarsveld and others 2005). The variation in provitamin A content of newly harvested roots, however, can be as much as 45%.

In western Kenya, for example, where vitamin A deficiency is a public health problem and white sweetpotato is an important secondary staple food, orange-fleshed sweetpotato was introduced and its consumption promoted along with that of other vitamin A-rich foods (Hagenimana and others 2001). Ten women's groups—all of whom were farmers—grew a number of sweetpotato varieties in on-farm, group plot, trials. Five groups received nutrition education, individual counseling, and participatory rapid appraisal techniques to promote vitamin A consumption, while the other 5 served as the control groups and received no additional promotion. Changes in the amount of vitamin A consumed by their children under 5 y old were assessed before and after 1 y of the program using the Helen Keller Intl. food-frequency method (Rosen and others 1993).

Roots were analyzed for β-carotene content in the form of boiled and mashed puree, sweetpotato flour, and processed products. Children in the intervention group consumed vitamin A-rich foods 93% more often than the control children, especially orange-fleshed sweetpotato, mangoes, dark-green leafy vegetables, butter, and eggs. The yields of orange-fleshed sweetpotato were at least twice those of white sweetpotato, as were the taste and appearance ratings. The dry matter content exceeded 25%. Beta-carotene values were such that 1 cup of boiled and mashed sweetpotato fed daily to children under 2 y old would meet their requirement for vitamin A, using the U.S. Inst. of Medicine's (2001) 12:1 β-carotene:retinol conversion. The authors concluded that orange-fleshed sweetpotato produced and prepared by women farmers can serve as a key food-based entry point for controlling vitamin A deficiency (Hagenimana and others 2001).

The Intl. Potato Center is working with national agricultural research institutes to breed provitamin A-rich sweetpotato in Ethiopia, Ghana, Kenya, Mozambique, Rwanda, South Africa, Tanzania, and Uganda. China, too, is breeding and promoting orange-fleshed sweetpotato in Sichuan province.

Africa's predominant sweetpotato cultivars are white- or yellow-fleshed varieties. The former contain no provitamin A, while the latter contain small amounts. The orange-fleshed varieties tend to have a high moisture content, while adults prefer varieties with a low water content. In East and Central Africa, preference is for varieties with > 27% dry matter, while in southern Africa varieties with 15% are acceptable.

About 40 varieties of sweetpotato high in both provitamin A and dry matter have been introduced to sub-Saharan Africa. Of these, 10 to 15 varieties are being tested widely in different agro-ecological areas in some countries (Table 4-1).

Besides β-carotene, orange-flesh (and both white and yellow flesh) sweetpotato roots are an important source of carbohydrate-energy, vitamin C, vitamin B_6, copper, potassium, iron, and fiber. The vitamin C and β-carotene may work as antioxidants to help eliminate free radicals, which are molecules that contribute to the damage of both cells and cell membranes and are associated with the development of conditions such as colon cancer, atherosclerosis, and heart disease (for example, Roomi and others 2005).

4.2.4 The expected dietary impact of vitamin A-enriched sweetpotato

To determine the expected dietary impact of consuming β-carotene-rich sweetpotato, 3 factors have to be considered:

Table 4-1—Sweetpotato varieties in Africa.

Variety	Color[a]	Source[a]	DM[b] (%)	Mean β-carotene mg/100 g fresh wt[b]	Source of roots assayed
Ejumula	Intermediate orange	Local landrace from Uganda	25 to 39	5590 to 15288	Tanzania, Uganda
Japon	Intermediate orange	CIP-introduced	23 to 34	2903 to 8887	Mozambique, Uganda
Jewell	Intermediate orange	American variety	24	14528	Tanzania
Jonathan	Orange	CIP-introduced	29	3634	Mozambique
Kala	Deep yellow	Local landrace from Uganda	37	183 to 1592	Uganda
Karoti Dar	Orange	Local landrace from Tanzania	31 to 36	2490 to 10281	Tanzania
Resisto	Deep orange	American variety	25 to 33	3140 to 17530	Mozambique, Tanzania
Simama	Deep yellow	Improved variety from Tanzania	42	73	Tanzania
SPK 004 –Kakamega	Orange	Kenyan improved variety	23 to 42	860 to 13336	Tanzania, Uganda
Tainung	Deep Orange	CIP-introduced	24 to 34	10570 to 17326	Mozambique, Tanzania
Zapallo	Deep orange	Tanzania/Uganda	20	1526	Tanzania

[a] CIP program reports; [b] DM = dry matter, values are ranges; values for β-carotene are ranges; Generose Mulokosi, Tanzania Food and Nutrition Center, personal communication, 2005.

retention, bioconversion, and intake levels (see Appendix 3). The HarvestPlus project's aim is to make available and encourage households in vitamin A-deficient areas to substitute orange-fleshed varieties for the white-fleshed sweetpotato currently eaten, thereby increasing vitamin A intakes.

The carotenoids in orange-fleshed sweetpotato comprise almost exclusively *trans*-β-carotene (Rodrigues-Amaya and Kimura 2004). The 13-*cis*-β-carotene and a few other unidentified carotenoids are present in much smaller amounts. *Cis*-β-carotene increases in boiled mashed sweetpotato and also in sweetpotato chips, but the level remains very low relative to *trans*-β-carotene. Table 4-1 shows the level of β-carotene in different varieties of colored sweetpotato.

True retention[2] of β-carotene has been assessed for the Resisto variety. Between 80% and 90% of the β-carotene was retained after boiling and mashing depending on the size of the root and boiling conditions—time and whether a lid was used (van Jaarsveld and others 2006).[3] K'Osambo and others (1998) observed similar results, although retention rather than true retention was measured. However, true retention after shade drying was 18% to 23% and after sun drying 9% to 15% (van Jaarsveld and others 2004).

In terms of bioconversion, a stable isotope study in humans found the relative vitamin A equivalency factors (β-carotene:retinol, wt:wt) for sweetpotato to be about 13:1 (Haskell and others 2004), which is similar to the current recommended vitamin A equivalency factor of 12:1 for β-carotene from plant foods (Inst. of Medicine 2001).

[3][(Carotenoid content per g of cooked food x g of food before cooking)/(nutrient content per g of raw food x g of food before cooking)] * 100

[4][Carotenoid content per g of cooked food (dry basis)/carotenoid content per g of raw food (dry basis)] * 100.

The per capita consumption of sweetpotato in different geographic areas around the world may not undergo any significant change in the short term. Governmental and nongovernmental promotion to substitute nonorange varieties with orange varieties may gradually increase the consumption of the β-carotene-rich varieties.

4.2.5 Sweetpotato as source of protein

As mentioned previously, sweetpotato is not a rich source of protein, and the quality of the protein is not high, particularly the content of tryptophan and sulfur amino acids (Ravindran and others 1995; Egnin and Prakash 1997; Prakash and others 1997; Shireen and Pace 2002). Consequently, human and animal diets that contain sweetpotato as a major component of the diet require inclusion of high-quality protein sources.

The introduction of the protein-enriched sweetpotato would provide an additional source of protein with improved quality as well as carbohydrates and micronutrients. The protein-enriched sweetpotato will have an important benefit for animal nutrition, given that sweetpotato roots are already a common constituent of animal feed in some countries (for example, China). In addition, the genetically modified protein-enriched ASP-1 sweetpotato was initially developed as a potential food for astronauts on long-term space missions. The improved protein content and high nitrogen bioavailability will not reduce undernutrition in developing countries if energy intakes do not increase currently because the protein will be converted to energy (more on the issue of protein-energy malnutrition can be found in Appendix 4).

Most of the proteins in sweetpotato roots are sporamin proteins with trypsin inhibitory activity that have a role in storage, plant protection (defense against pathogens), and regulation of endogenous protein-degrading enzymes (Shewry 2003). Trypsin

inhibitor activity in roots impairs protein digestibility. Boiling roots reduces the trypsin inhibitor activity and thus improves protein digestion (Dominguez 1992). In addition, some studies show that ensiling can be an energy-efficient alternative for this purpose (Peters and others 2002).

Whereas sweetpotato roots are low in protein, the green parts (vines) are a good source, but have the drawback of being high in fiber. Therefore, vines can only be fed in limited quantities to nonruminants. In addition, both root and vine proteins are deficient in essential amino acids, particularly tryptophan, but also in lysine and sulfur amino acids such as methionine (Dominguez 1992; Egnin and Prakash 1997; Prakash and others 1997; Dapeng and Xiu Qing 2004). Therefore, protein supplements are needed in sweetpotato-containing animal diets. Given that root protein content is inversely related to dry matter content, conventional breeding for high protein is difficult (Dapeng and Xiu-Qing 2004) and may conflict with the preference of some consumers, including Africans, for high dry matter content. Nevertheless, 2 sweetpotato lines with low trypsin inhibitor and high protein levels in roots are being developed by conventional means for animal feed and human food applications (Toyama and others 2006).

Improving both the protein quantity and quality in sweetpotato roots would thus benefit animal nutrition and diminish or obviate the need for supplementation with high-protein feed ingredients.

4.2.6 Expected dietary impact of ASP-1 modified sweetpotato

Genetic modification for nutritional purposes has helped to improve the nutrient content of certain foods (see other case studies). Despite the potential benefits of this approach, such foods are yet to be introduced into commerce and specifically in countries where they would be most effective. The ASP-1 protein-modified sweetpotato was engineered to improve the quality of protein in sweetpotato, which can be of benefit to animal diets that are high in sweetpotato and human diets.

In addition to the safety considerations, 2 main factors have to be considered to determine the expected dietary impact of using ASP-1 modified sweetpotato—acceptability and intake levels. Protein-energy malnutrition results when the diet lacks energy and macronutrients. Protein-rich sweetpotato could be part of a strategy against undernutrition in the long term if total energy intake increases. In terms of acceptability, the dry matter content is likely to be an important factor because many consumers prefer a high content.

4.2.7 Antinutrients in sweetpotato

Compositional analysis is part of the safety assessment of GM crops in general and commonly includes a range of nutrients, antinutrients, and important secondary metabolites that can be measured in roots and leaves. Unlike many major crops, there is no OECD consensus document to guide the composition analysis for sweetpotato.

Sweetpotato contains a number of antinutrient compounds that have been well described, including oxalic acid, trypsin inhibitor, and furanoterpenoid compounds (Woolfe 1992; Shireen and others 2001). Oxalic acid is an organic acid that can complex with minerals during intestinal passage and may, therefore, alter their bioavailability. Stress conditions have been reported to lead to increased levels of oxalic acid in sweetpotato roots (Woolfe 1992). Trypsin inhibitors in sweetpotato, also designated sporamin, make up a large proportion of the soluble proteins (60% to 80% depending on the variety). The number of different molecules and their characteristics (for example, susceptibility to heat and pH) differ among sweetpotato cultivars (Scott and Symes 1996). Heating, such as in cooking, removes some, but not all trypsin inhibitor activity (Woolfe 1992; Sasi Kiran and Padmaja 2003).

Testing for antinutrients is not routinely done in variety testing, which focuses on productive traits such as yield and dry matter. However, measurement of antinutrient levels is both common and important in the comparative safety assessment of novel crops, such as GM crops. Shireen and others (2001) analyzed the composition of ASP-1 sweetpotato, specifically macronutrients, amino acids, and trypsin inhibitor, as part of a nutritional study in hamsters.

Levels of secondary metabolites such as furanoterpenoids (for example, ipomeamarone) may be negligible in nondiseased sweetpotato, but elevated levels can be found in sweetpotato plants suffering from insect or mold infestation. Ipomeamarone and related compounds cause liver toxicity in experimental animals (Wilson and others 1970). Furthermore, molds growing on diseases sweetpotato convert these furanoterpenoids to compounds, such as ipomeanol, that exert cytochrome-P450-mediated toxicity on lungs, kidney, liver, and other organs of various animals, and on liver of humans (Woolfe 1992; Lakhanpal and others 2001). However, there are no reports of human adverse reactions to sweetpotato, probably because most diseased sweetpotato are discarded due to the bitter taste of furanoterpenoids, and because the levels of these compounds are decreased by cooking or baking (Woolfe 1992).

Box 4-2—Orange-fleshed sweetpotato
- Rich in β-carotene compared with white- and other colored-fleshed types.
- Obtained through conventional breeding.
- Promoted by HarvestPlus to control vitamin A deficiency, especially in Africa.
- Breeding program at CIP aimed at combining high β-carotene levels with high dry matter content and disease resistance.
- Range of varieties available for further breeding by local communities.
- History of safe use.
- Known to contain a number of antinutrient compounds that have been well described, including oxalic acid, trypsin inhibitor, and furanoterpenoid compounds.
- Efficacy has been determined in a human study in South African school children.

4.3 Orange-Fleshed Sweetpotato

4.3.1 Crop characteristics

4.3.1.1 Breeding and agronomic performance of orange-fleshed sweetpotato. Plant breeding is ongoing with provitamin A–rich orange sweetpotato varieties to improve the sensory characteristics (higher dry matter content) and, at the same time, improve the resistance to viruses and environmental stress conditions such as drought (Box 4-2). It should be noted that sweetpotato is a polyploid (that is, it has more than 2*n* chromosomes), which makes breeding traits into sweetpota-

to difficult due to incompatibility, low seed setting, and sterility.

The Intl. Potato Center (CIP) considers any sweetpotato having at least 100 μg β-carotene per gram of fresh root as being β-carotene rich. For HarvestPlus, this trait must be combined with a high dry matter content and no loss of any agronomic traits for the variety to be considered biofortified (Nestel and others 2006).

Efforts to develop new sweetpotato clones with high dry matter and high β-carotene were initiated in 1996 at CIP's headquarters in Lima, Peru. More than 100 different sweetpotato lines were used as parental materials. In all, more than 150000 offspring were developed and subjected to 5 seasons of recurrent selection and evaluation. Of these, 100 superior clones were selected for shipment overseas in 2002. By the breeder's standards, all are considered to be high yielding, high in β-carotene, and to contain sufficient dry matter to meet local taste preferences. Because of significant clonal variation, farmers will have options when selecting plants suited to local conditions. In terms of safety, all the breeding material is based on clones that are already consumed.

4.3.1.2 Maintaining desirable characteristics. Beta-carotene content is inversely related to dry matter content (Manrique and Hermann 2001). Breeding efforts have focused on increasing the dry matter content through conventional breeding methods using varieties with a higher dry matter content. The CIP breeders are using recurrent selection in an attempt to break the linkage between high dry matter and β-carotene content so that these 2 traits can be combined. Parental clones are induced to flower by grafting onto *Ipomoea nil*. All selected clones are thoroughly evaluated for fresh yield, β-carotene and dry matter contents, resistance to root knot nematode (*Meloidogyne incognita*), and virus diseases (www.harvestplus.org).

4.3.1.3 Synthesis pathway of β-carotene. The key genes and enzymes involved in β-carotene synthesis have not been described for sweetpotato, but the pathway seems to be similar in plants, for example, in *Arabidopsis* and in bell pepper. The biosynthesis of β-carotene in plants is described in Appendix 2. The molecular mechanism responsible for the increased and variable levels of β-carotene synthesis in the orange-fleshed sweetpotato varieties compared with other differently colored varieties is still unknown. This is not uncommon in that the precise molecular mechanisms underlying the desirable characteristics obtained through conventional breeding are not known in most cases.

4.3.1.4 Stage of product development—unique questions/challenges. One variety of orange-fleshed sweetpotato has been shown to be an efficacious source of provitamin A among South African school children (see section 4.3.3.1). HarvestPlus is currently supporting research to assess the efficacy of daily consumption of boiled or fried orange-fleshed sweetpotato to improve the vitamin A status of nonpregnant, nonlactating Bangladeshi women, and to estimate the relative vitamin A equivalency of β-carotene from the 2 different preparations of orange-fleshed sweetpotato (boiled or fried).

Sweetpotato is currently a secondary crop, not the main staple food, in the diet of people living in East and Southern Africa. To increase orange-fleshed sweetpotato consumption, 2 criteria must be satisfied: sufficient planting material must be available and the material must have both the agronomic traits farmers desire and the sensory traits (color and dry matter content) consumers want. As stated previously, a number of sweetpotato varieties have been selected for further optimization in collaboration with local farming communities. Improved varieties will be promoted through national agricultural and public health nutrition programs.

4.3.2 Safety assessment (provitamin A sweetpotato)

4.3.2.1 History of safe use. Yellow- and orange-fleshed sweetpotatoes are consumed around the world, in both developed and developing countries.

4.3.2.2 Compositional changes. High β-carotene concentration and dry matter content are the targeted selection goals for sweetpotato programs. In changing the β-carotene content, it is possible that the carotenoid profile may also change.

The pathway leading to carotenoid biosynthesis has been well studied in plants and microorganisms (Cunningham and Gantt 1998; Sandmann 2001; Taylor and Ramsay 2005). Data on the pathway in sweetpotato are limited to analyses of carotenoids and their intermediates in differently colored sweetpotato. The results indicated that as the carotenoid level increases, the diversity in the types of carotenoids decreases, with β-carotene becoming the predominant carotenoid (Woolfe 1992).

Products of conventional breeding have not traditionally been subjected to the extensive compositional analysis, but their safety is supported by a history of safe use.

4.3.2.3 Metabolism of the target nutrient. The metabolism of β-carotene in humans and animals is considered in Appendix 3 and in section 5.5.3 of the Golden Rice 2 case study chapter.

4.3.2.4 Unintended changes. Unintended changes are not an occurrence specific to genetic modification; they may also occur in conventional plant breeding (Cellini and others 2004). Most of the reported analyses of sweetpotato compare the carotenoid, β-carotene, and dry matter contents of various sweetpotato varieties. No unexpected changes have been reported during conventional breeding in this species. In more general terms, unintended effects may occur during breeding, and plants showing unwanted changes are likely to be removed from the breeding program, which in many cases will depend on the breeder's choice and remain nondocumented in public literature.

No metabolic pathways were intentionally changed in the orange-fleshed sweetpotato varieties, which otherwise could have been the target of an analysis of potential changes. Since no foreign genes were introduced into the sweetpotato during the traditional breeding processes, the safety assessment normally applied to GM crops does not apply. The new varieties are assumed to have similar metabolic pathways to those in their parents, although the content or activity of certain enzymes could have been changed in the new varieties compared with their parental plants.

4.3.3 Nutritional assessment

4.3.3.1 Bioavailability of β-carotene. An efficacy trial has been conducted among 5- to 10-y-old South African children fed 125 g of boiled and mashed orange-fleshed sweetpotato (1031 μg retinol activity equivalents per day as β-carotene) or an equal amount of boiled and mashed white-fleshed sweetpotato devoid

of β-carotene for 53 school days. Compliance was 90% in both groups. Using the modified dose–response test, the study showed an improvement in vitamin A liver stores of the treatment group relative to the control group (van Jaarsveld and others 2005).

An earlier study by Jalal and others (1998) showed that serum retinol concentrations markedly improved when dewormed Indonesian children (5 to 10 y old) consumed meals containing β-carotene-rich sweetpotato (750 or 200 retinol equivalents per day) and fat (27 compared with 12 g/d) for 3 wk.

A recent stable isotope study among Bangladesh men, however, found that daily supplementation with 750 μg/d of RE from sweetpotato for 60 d did not alter vitamin A stores (Haskell and others 2004). The authors postulated that the food preparation method (no fat) may have influenced such results because dietary fat may influence the uptake of β-carotene.

Bioavailability studies on β-carotene from orange-fleshed sweetpotato have not been carried out in domestic animals. It is known that β-carotene taken up from fortified animal feeds can be present in edible animal products, such as chicken eggs and dairy milk. For example, Woolfe (1992) cited several earlier studies showing that feeding dried roots of orange-fleshed sweetpotato to led to the pigmentation of egg yolk of poultry, as well as to increased levels of β-carotene and vitamin A in cow's milk.

Although the main focus of this chapter is on the human food use of the orange-fleshed sweetpotato, a major part of the sweetpotato production—including roots and vines—goes into animal feed, particularly in China (see section 4.2.2). Bioavailability studies with domestic animals usually measure the uptake of β-carotene from ingested feedstuffs into serum or liver (Lee and others 1999). The efficiency with which β-carotene is taken up and mobilized into serum varies with animal species, with preruminant calves being considered efficient. However, the cost of this animal model often precludes its use and swine or Mongolian gerbils are used instead.

4.3.3.2 Animal performance and nutritional wholesomeness of β-carotene-rich sweetpotato. The orange-fleshed sweetpotato have not been intended as a nutritionally enhanced animal feed per se, but this may change depending on, for example, a shift in the focus of programs promoting the sweetpotato.

Woolfe (1992) provides an overview of the animal feeding trials that have been carried out with sweetpotato. Nutritional requirements also vary within an animal species, depending on the phase of life such as lactation or weaning. For pig feeding, sweetpotato and derived starch commonly serve as energy source. Performance and digestibility feeding studies show that maize in pig diets can be replaced partially or wholly by sweetpotato, while the same also holds true for starches derived from cereals and from sweetpotato. Feeding studies in poultry show that sweetpotato can at least partially replace the maize component of chicken diets without adversely impacting nutritional performance (for example, body weight gain, feed efficiency) and meat or egg production. For pigs and poultry, consideration should be given, though, to the possible effects of trypsin inhibitors and crude starch on digestibility, which may be prevented by appropriate processing techniques (Woolfe 1992).

Fermented sweetpotato roots and vines are reported to be used as animal feed, as are byproducts of industrial processing of sweetpotato, such as canning, distillation, and starch production, which may be more common in developed countries (Woolfe 1992). Beta-carotene itself is also used as feed additive in some species, such as horses, because of its reputed stimulating effect on reproductive functions (Kienzle and others 2003).

Box 4-3—Protein-enriched sweetpotato
- Rich in protein compared with conventional types.
- Obtained through insertion of *asp-1* gene.
- Aimed at improving the nutritional quality of protein in sweetpotato.
- Known to contain a number of antinutrient compounds that have been well-described, including oxalic acid, trypsin inhibitor, and furanoterpenoid compounds.
- Composition studies show changes in protein levels but no other critical changes.
- Feeding studies on nutritional properties show no adverse effects in animals compared with conventional sweetpotato.
- May be useful in certain populations, but efficacy is not yet proven on human populations.

4.4 ASP-1-containing Sweetpotato

4.4.1 Crop characteristics

Box 4-3 provides a summary of the characteristics of protein-enriched sweetpotato. The engineering of sweetpotato varieties was aimed at increasing the quality of protein for use as a food for astronauts, but may also add value for other purposes, such as food and animal feed, in various developing countries and some southern U.S. states where it is grown.

4.4.1.1 Origin of the *asp-1* gene. The protein ASP-1 is produced from a novel, synthetic transgenic storage protein gene (*asp-1*) coding for a storage protein rich in essential amino acids (EAA, 80%) with improved protein stability in plants. The coding sequence of the synthetic *asp-1* gene is 284 bp long (Prakash and others 1997) The primary sequence consists of 4 repeats of an amino acid sequence, linked by short regions (Figure 4-2) that favor protein folding to produce a tetramer-like structure similar to the plant storage proteins zein and phaseolin (Kim and others 1992).

4.4.1.2 Introduction of the *asp-1* gene. The *asp-1* gene was linked to 35S CaMV promoter and Kozak translation enhancer (Espinoza and others 1989; Egnin and Prakash 1995) and *nos* terminator (Egnin and Prakash 1995) and inserted using *Agrobacterium tumefaciens*-mediated transformation into sweetpotato petioles. The vector DNA also included an *nptII* gene (with *nos* promoter and terminator) present at the right border and a *gus* gene (35S-GUS-nos) at the left border of the transfer-DNA (Figure 4-3). A number of different events were produced. Selection was achieved via the *nptII* gene, and the resulting transformants were screened by polymerase chain reaction and southern blot analyses for those that contained *asp-1* and did not include the *gus* gene. The expression of the *asp-1* gene in multiple tissues, including roots and leaves, was confirmed by northern and western blots (Egnin and Prakash 1997; Prakash and others 1997). The lead event is TA3 and is the event that is discussed in this case study. The insert in this event has not been characterized with regard to copy number, or the

presence or absence of additional elements from the transformation vector.

4.4.1.3 Agronomic performance and field trials. The 1st trials were conducted at Tuskegee Univ. in 1997, using a split plot design with 5 GM varieties, the isogenic line, and commercial varieties. This was repeated at Tuskegee in 1999 and in the Virgin Islands in 2000 (Egnin and others 2001a, 2001b).

Agronomic trials were also conducted at Tuskegee in row plots in 1998, 2001, 2002, 2003, and 2004 to establish yield stability and expression levels of ASP-1. In addition, hydroponic studies were conducted to establish the expression pattern for ASP-1 and the major native storage root proteins (sporamin and β-amylase; [Egnin and Prakash 1997; Egnin and others 1998, 2002, 2003]).

The yield of the TA3 sweetpotato is comparable to that of the isogenic conventional sweetpotato (Egnin and others 1998, 2001a, 2001b). The mean yield of the TA3 was not statistically different. In addition, the agronomic characteristics of TA3 were equivalent to the comparator—there were no significant differences in growth pattern, photosynthetic efficiency, or other characteristics.

4.4.1.4 Stage of product development—unique questions/challenges. The U.S. Natl. Aeronautics and Space Administration at the Tuskegee Univ. Center for Plant Biotechnology and Genomic Research supported this project as part of a study to develop crops for use in space exploration. The protein-enhanced sweetpotato has been grown in hydroponic culture and tested in numerous field trails. In addition, there are a number of studies on the protein content and composition of the modified crop.

To increase consumption of protein-enriched sweetpotato, sufficient planting material must be available. This could be achieved by tissue culture propagation or increases from vines. No studies of sensory traits of sweetpotato with increased protein content have been carried out in humans.

4.4.2 Safety and nutritional assessment of the ASP-1-containing sweetpotato

4.4.2.1 History of safe use. Derived from the synthetic *asp-1* gene, ASP-1 protein is a novel protein not previously consumed by humans. However, ASP-1 has similarity to known crop seed storage proteins. Bioinformatic analyses revealed no homology to any known allergen (Egnin and Prakash 1997; Braithwaite 2001). The results show that ASP-1 protein shares the helix-turn-helix motif with other storage proteins and is homologous to a barley storage protein. This barley protein has transcription activator activity and such an activity could be the cause of increases in the expression of β-amylase and sporamin in the TA3 event. ASP-1 protein has some sequence similarity to other artificial and natural storage proteins and it is structurally similar to a tryptophan repressor, which blocks access to promoters by RNA polymerase, although the primary amino acid sequences have no significant similarities.

In vitro digestibility data, which is a common part of regulatory assessments of the potential toxicity and allergenicity of transgenic proteins, is not available for the ASP-1 protein. The Intl. Life Science Inst. (ILSI) recently standardized the pepsin digestibility assay protocol in a multilaboratory evaluation (Thomas and others 2004), and this should be taken into account if this assay is carried out on the ASP-1 protein.

The safety of the purified ASP-1 protein has not yet been tested for its potential acute toxicity in laboratory animals. Given the novelty of the protein, it is conceivable that, depending on the outcomes of the *in vitro* and *in silico* studies with ASP-1, regulatory agencies may require *in vivo* oral studies with purified ASP-1.

To test nutritional effects, small-scale feeding studies with storage roots were carried out in hamsters, as a mammalian model. Examination of the hamsters' brain, liver, and kidney weights; plasma and liver lipids; and bone calcification showed that GM lines did not identify any adverse effects compared with animals fed diets containing conventional sweetpotato (Shireen and Pace 2002; Shireen and others 2002).

4.4.2.2 Plant expression of the introduced genes. Analysis of expression levels of transgene products is a common feature of the safety assessment of GM crops. In addition, it enables estimation of consumers' exposure level to this new protein. The levels of ASP-1 have not been determined with accuracy because of the lack of a validated Enzyme-Linked ImmunoSorbent Assay (ELISA) method. However, the ASP-1 protein is detectable in the storage roots and shoots of sweetpotato containing the TA3 event and is present in the expected tetrametric form. This size is seen in both native and denaturing western blots.

The GM plants contain the *nptII* antibiotic resistance marker gene, which was used for selection. This protein is recognized as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (Redenbaugh and others 1992; Fuchs and others 1993). The amount of NPTII protein has been determined in the sweetpotato leaves (Jaynes and others 1985) but not in the roots. A qualitative ELISA is commercially available, but there is no commercial standard against which to quantify the results. Because of this, the quantification of native and transgene proteins was carried out through equal weight (sweetpotato storage roots) and equal extraction volumes.

MLEELFKKMTEWIEKVIKTMGPGRMLEELFKKMTEWIEKVIKTMGPGRMLEELF
KKMTEWIEKVIKTMGPGRMLEELFKKMTEWIEKVIKTM

Figure 4-2—Primary structure (amino acid sequence) of ASP-1 protein.

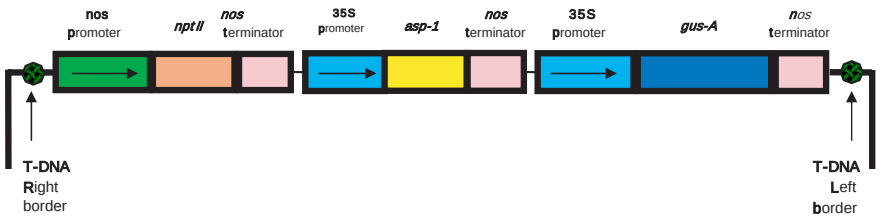

Figure 4-3—Vector used in production of sweetpotato event TA3 (2006, personal communication from M Egnin; unreferenced).

4.4.2.3 Compositional changes. Proximates were analyzed in samples obtained from field trials that had been carried out on split plots for 4 y. These analyses showed that dry matter, ash, total carbohydrate, and crude fat were slightly increased, while the amino acids lysine, tryptophan, methionine, threonine, and isoleucine were significantly increased, up to manyfold for tryptophan, over the control samples. In addition, 2- to 3-fold increases in sucrose, glucose, and fructose levels were observed (Egnin and Prakash 1997; Egnin and others 1998, 2001b, 2002; Shireen and others 1999, 2001; Shireen and Pace 2002). In addition to ASP-1 protein being produced, quantities of sporamin protein were also observed in storage roots grown in the field and β-amylase was increased about 2-fold; thus, total protein was increased 3- to 5-fold. The same effects were found in sweetpotato roots grown in hydroponic cultures. In addition, protein levels were increased 2- to 3-fold in the leaves. The increased total protein content of roots is thus primarily due to the enhanced levels of native proteins, especially sporamin in the storage roots (Egnin and others 2002), in addition to ASP-1. It is not clear what the mechanism for this increase is, although homology of ASP-1 to a barley storage protein with transcription activator activity might be implicated.

4.4.2.4 Unintended changes. An unintended effect of expressing the *asp-1* gene in sweetpotato is an increase in native protein concentration in the storage roots, particularly the major proteins sporamin and β-amylase, and a general increase in protein concentration in leaves. The exact amount and nature of these changes in leaves have not yet been determined. Sporamin is the main protein present in sweetpotato roots and has some possible trypsin inhibitor activity (Hattori and others 1985; Hou and others 2001; Shireen and others 2001). Sporamin shares significant amino acid sequence identity with some Kunitz-type trypsin inhibitors, and has been shown to have trypsin-inhibitor type activity (Yeh and others 1997). Heating, such as cooking, may not remove all trypsin inhibitor activity (Woolfe 1992; Sasi Kiran and Padmaja 2003). However, hamster feeding studies did not show any deleterious effects of consuming raw sweetpotato flour. As described previously, changes in dry matter and sugar content were also observed. Chakraborty and others (2000) found similar effects on protein concentration in potato (*Solanum tuberosum*) using an amaranth protein gene. Studies of cassava expressing ASP-1 were comparable to those in sweetpotato in terms of protein level increases (Zhang and others 2003). Thus, the observation of such increases in protein in plant storage tissues is not restricted to this crop and protein gene, but may be a more generalized phenomenon.

The phenotypic and agronomic properties of selected cultivars are also powerful indicators of the presence or absence of unintended changes and, as stated previously, the TA3 event showed no changes in agronomic characteristics (see section 4.4.1.3). In addition, as with conventional breeding, plants showing unwanted changes are removed from the program and remain nondocumented in public literature.

Several antinutrients were not determined in this sweetpotato event (Shireen and others 2001).

In hypothesis, the general increase in protein caused by the modification might also have raised levels of any intrinsic allergens of sweetpotato. However, sweetpotato is generally considered as being rarely allergenic or nonallergenic, and reported cases of allergy are rare. For example, allergic reactions following consumption of sweetpotato by a limited number of patients were recently reported for the first time (Velloso and others 2004). In addition, there are no known accounts either of the storage protein sporamin being an allergen.

4.4.2.5 Metabolism and nutrition of the target nutrient. Proteins are distinct from other classes of nutrients in terms of their size, digestibility, and metabolism. Most dietary proteins are too large to be absorbed intact from the gastrointestinal tract and are generally denatured by stomach acid and then digested by exo- and endoproteases, such as those excreted into the stomach and into the small intestine from the pancreas. The resulting amino acids and short peptides are taken up by the mucosa, and amino acids are translocated into the portal vein. The overwhelming majority of dietary proteins, therefore, possess no potential for systemic toxicity and no inherent hazard.

The expected endpoint of increased protein content and quality is improved human nutrition and health. The protein content in TA3 is increased approximately 67% over the existing lines. The levels of five of the most limiting essential amino acids (lysine, tryptophan, methionine, threonine, and isoleucine) increased, as did dry matter and sugar content.

Tryptophan is the most limiting amino acid and is almost absent in sweetpotato. The TA3 material has about 100-fold the amount of tryptophan than in conventional lines. To illustrate the potential benefit, the amount of sweetpotato that would be required to meet daily protein requirements could decrease from 5 kg to 300 g. However, one should keep in mind that sweetpotato is not the only food source of proteins. The appropriate comparator for feeding studies with the TA3 material is a diet containing another source of protein.

The results of a 28-d Syrian golden hamster feeding trial (10 animals/treatment) showed that the GM diet using freeze-dried flour was palatable, and the increased protein and amino acids were bioavailable to the hamsters. The animals fed TA3 sweetpotato had no negative indicators compared with those fed conventional sweetpotato (brain, liver, and kidney weights) (Shireen 2002). Hamsters fed with ASP-1 sweetpotato flour exhibited equal total cholesterol, triglycerides, and LDL-cholesterol levels in their plasma and liver when compared with those in the conventional control group. The ASP-1 sweetpotato showed superiority in true protein digestibility, net protein utilization, and biological value of the protein relative to the conventional control.

Thus the GM diet was palatable to the animals and the increased protein and amino acids were bioavailable to the hamsters. However, it has not been shown that these proteins are digested and made bioavailable to humans—a study using simulated gastric and intestinal fluid could address this question.

4.5 Discussion of Differences and Similarities

These sweetpotato crop case studies demonstrate 2 types of modifications that may be applied to a crop using different approaches. As both these improved nutrition sweetpotatoes are expected to offer benefits to the target populations, considering both the risks and benefits and weighing these factors against each other should be part of the risk analysis process for which the safety and nutritional assessments serve as inputs (FAO/WHO 1997).

In addition, it is important to consider the beneficial increase in the intake of key nutrients, such as β-carotene and protein, due to the introduction of the modified varieties that substitute for local varieties.

Compositional and nutritional data are available and show that the orange-fleshed sweetpotato can be a good source of β-carotene in locations where the human population consumes sweetpotato. High-protein sweetpotato has been shown to be an excellent source of protein in hamster feeding studies.

There are a number of studies already completed on the safety and nutritional assessment of the ASP-1 protein itself and in the GM sweetpotato. Molecular and animal studies have not identified safety concerns.

4.6 Recommendations

The preceding sections summarized the information regarding the conventionally bred orange-fleshed sweetpotato and the transgenic ASP-1 protein-enriched sweetpotato. It is noted that a dichotomy exists with regard to the regulatory requirements for both types of crops, with extensive data on molecular characteristics, comparative compositional analysis, and the safety of transgene products being required for the GM product. It needs to be seen in this light that the following recommendations make a distinction between the requirements of the orange-fleshed and the ASP-1 sweetpotato.

For the orange-fleshed sweetpotato, the following recommendation is made.

Recommendation 1. The promotion of orange-fleshed sweetpotato described previously is not specifically geared toward animal nutrition, but to human nutrition. A number of studies testing the nutrition of this sweetpotato in target human populations have been carried out. There is no specific requirement to additionally test the nutrition of this sweetpotato in domestic animals. However, it can be envisaged that in the future, orange-fleshed sweetpotato might also be promoted for certain purposes in animal feed. If testing is useful, for example because of lack of experience with a new application in animal feed, the choice of the appropriate animal model will depend upon the target animal and the purpose of feeding this sweetpotato to these animals. Guidance on animal trials is provided by a report authored by an ILSI task force and entitled *Best Practices for the Conduct of Animal Studies to Evaluate Genetically Modified Crops.*

For the ASP-1 sweetpotato, the following recommendations are made.

Recommendation 1. The safety of the genetic modification with the *asp-1* gene and derived ASP-1 protein should be assessed in the ASP-1-containing crop, including the characteristics of the introduced and other affected proteins. This may entail studies supplementary to those that have already been performed.

Recommendation 2. Compositional studies have been carried out and should be examined for completeness. Supplementary compositional analysis studies may consider, for example, appropriate antinutrients, such as oxalic acid, trypsin inhibitor, and others, where appropriate. It is noted that for other crops, OECD has developed consensus documents with key parameters for compositional analysis of new crop lines. However, such a consensus document on sweetpotato has not yet been published.

Recommendation 3. The phenotypic properties of the sweetpotato line should be assessed when grown in representative production sites, as part of a comparative safety assessment. Again, data exist for these parameters. The studies would ideally compare the test sweetpotato with non-GM varieties. Based on any differences identified, further studies may be needed to investigate the safety and nutritional properties of the enriched crop.

Recommendation 4. Performance of animals fed ASP-1 sweetpotato compared with those fed conventional sweetpotato varieties could be considered using a suitable animal model, for which an ILSI task force, in a report titled *Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Output Traits*, has formulated guidance (ILSI 2007). For bioefficacy of the proteins in the enhanced protein sweetpotato, a hamster study that gives some indication in this area is available.

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Chapter 5: Golden Rice 2

ABSTRACT: Golden Rice 1 was developed to help control vitamin A deficiency (VAD). To construct Golden Rice 2, the phytoene synthetase gene (*psy*) from maize and the carotene desaturase gene (*crtI*) from *Erwinia uredovora* were inserted into rice. Evaluation of phytoene synthase (the rate limiting step in carotenoid biosynthesis) from several plant sources led to the identification of the *psy* gene from maize as the most efficacious source, resulting in the greatest accumulation of total carotenoids and β-carotene. Golden Rice 1 contains about 1.6 μg of total carotenoids per gram of dry weight of grain. Golden Rice 2 contains as much as 37 μg total carotenoids per gram of dry weight of grain, of which 31 μg/g is β-carotene. While the quantity of β-carotene is high, its bioavailability is unknown. Golden Rice 2 was developed with the expectation that it could make a major contribution to the vitamin A requirement. It is conservatively estimated that a breastfed 1- to 2-y-old child could derive 60% of the of U.S. recommended dietary allowance (RDA) from the consumption of approximately 70 g of uncooked Golden Rice 2; an average serving size for a child this age in Thailand, for example, is 160 g. The higher level of β-carotene raises the possibility that Golden Rice 2 may help reduce many of the deaths attributed to VAD (Hess and others 2005). Biofortified rice with high levels of β-carotene is in an early stage of development. The published data provide a description of the DNA construct introduced into rice and report the concentrations of both total carotenoids as well as that of the 5 major carotenoids present in representative transgenic rice plants. The safety assessment of Golden Rice 2 follows generally accepted international guidelines. Beta-carotene biosynthesis is a peripheral biosynthetic pathway that diverts very small amounts of biosynthetic precursors from plant metabolism and would, thus, not be expected to produce major changes in composition. Hence, the safety assessment can be targeted to the impact of the insertion on cellular carotenoids and other metabolically related compounds. Plant phenotype, seed weight, and germination were not affected by the presence of the genetic modification. Apart from safety concerns, there exists an urgent need for studies to evaluate the efficacy of this product as a bioavailable source of β-carotene in animals and humans. Proof of efficacy would allow Golden Rice 2 to be widely distributed. Appropriate safety assessments are needed and should at least include (1) characterization of the inserted DNA, (2) determination of the composition according to OECD consensus documents, (3) analysis of the carotenoid metabolite pool, and (4) evaluation of the efficacy of this product as a source of β-carotene (although it is not related to safety). The safety of carotene desaturase gene (*crtI*) and its products from *E. uredovora* requires characterization because it has no history of safe use in foods.

5.1 Introduction

Golden Rice 1 stands as a prototype that demonstrated the feasibility of using rice as a means to provide vitamin A precursors to populations in which rice is the staple food (Ye and others 2000). However, replacing regular rice with Golden Rice 1 might not increase β-carotene intakes to the level of the total daily requirement for vitamin A (Table 5-1, Dawe and others 2002; Bouis and others 2003; Zimmerman and Qaim 2004). Table 5-1 assumes that the conversion of β-carotene to vitamin A occurs with a ratio of 12:1 (pessimistic) to 6:1 (optimistic). In the best-case assumption, Golden Rice might improve vitamin A intake by 20% to 60% of the RDA. Preliminary studies in 2 human subjects showed a bioavailability/bioconversion of Golden Rice β-carotene to vitamin A in the range of 3:1 to 4:1 (2007 personal communication from R Russell, unreferenced), which would make Golden Rice effective in reducing or eliminating most of the VAD's adverse effects. This assumes that 100% of daily rice intake is converted to Golden Rice. Economic analysis using conservative assumptions indicates that, while not a panacea, Golden Rice would save lives more economically than the interventions that are presently in use (Stein and others 2006). This conclusion has recently been challenged (Krawinkel 2007) and the researchers have responded that while Golden Rice would be more efficacious than currently used interventions, it is intended to supplement existing efforts rather than supplant them (Stein and others 2007). Golden Rice 2 (Paine and others 2005), reported in this case study, was constructed using the same genetic strategy applied in the construction of Golden Rice 1, with the exception that the phytoene synthase gene from *Zea mays* replaced the gene isolated from *Narcissus pseudonarcissus*. Golden Rice 2 contains approximately 20-fold more carotenoids than Golden Rice 1 and is, thus, a more promising source of vitamin A for rice-consuming populations, which would not require that all rice consumed be Golden Rice.

5.2 Rice in Human Nutrition

5.2.1 Host crop

Rice is the world's most important cereal crop for human nutrition. The earliest evidence of rice consumption dates back approximately 5500 y in Thailand (www.therice.org/rice/story.html), about 5000 y ago in India, and over 3000 y ago in China. Wet paddy cultivation is the predominant production technique, and more than half of the water used for agriculture is devoted to rice (www.fao.org). Rice cultivation is a major agricultural activity in many Asian countries. For example, more than 50% of the population of China, Thailand, and Vietnam engages in or directly depends on local rice agriculture. In Asia, 31% of all food energy consumed is from rice (country range = 2% to 71%); in Africa, 8% of energy intake is from rice (country

range = 3% to 47%); in Latin America, 11% of all food energy is from rice (country range = 1% to 30%). The contribution of rice to energy intake in North America, Europe, and Australia–New Zealand is negligible (<2%; IRRI 2005). Additionally, rice consumption varies among countries and within a single country among demographic groups; for example, affluent urban dwellers usually consume less rice per capita than poor rural populations do (Regmi and Dyck 2001). Although some rice is cultivated in Africa and the Americas (the United States is the 3rd leading rice exporting nation in the world), the majority of the rice consumed in these regions is imported (IRRI 2005).

5.2.2 Uses and nutrient content of rice in food and feed

Rice is used primarily for human food although small amounts of brown rice, rice bran, rice polishings, and rice hulls are used in animal and poultry feed (OECD 2004). White (polished) rice is not fed to animals because of its high cost. Brown rice contains approximately 74% available carbohydrate, approximately 7.5% protein (limiting amino acids = lysine and threonine), approximately 2% lipid, and 3% to 4% fiber. Brown rice provides significant quantities of thiamin, riboflavin, and niacin (www.nal.usda.gov/fnic/foodcomp/search; USDA Natl. Nutrient Database for Standard Reference, Release 19). A 100-g serving provides 1455 kJ. Polished white rice contains approximately 78% available carbohydrate, approximately 6.9% protein, approximately 1.4% fiber, and < 1% lipid. A 100-g serving of white rice provides the same amount of energy as brown rice; however, polishing removes > 70% of the niacin, thiamin, and riboflavin and reduces the iron content from approximately 2.5 μg/100 g by a similar percentage to < 0.6 μg/100 g. Beriberi (thiamin deficiency) can occur in Asian countries among people whose diets consist primarily of white rice (FAO 2001). Iron deficiency is common in populations for whom rice is the primary staple food (FAO 2001). Enriched white rice is fortified with iron, thiamin, and niacin (and with folic acid in the United States since January 1998) to control beriberi and iron deficiencies. Enriched rice, however, is of little nutritional benefit to the large numbers of rice consumers who grow their own rice or eat locally produced rice. Polished white rice is not a significant source of the fat-soluble vitamins A, D, E, and K. The OECD has published a consensus document on key food and feed nutrients and antinutrients found in rice (OECD 2004).

5.2.3 Expected geographic distribution and nutritional impact

VAD is widespread in South and Southeast Asia (Figure A1-1), which are the major rice-eating regions of the world. Sub-Saharan Africa, Central America, and parts of the northern portions of South America also have significant VAD. Rice contributes significantly to the diet in parts of these regions, including people who are most at risk of VAD. If nutritionally viable and acceptable to consumers, Golden Rice 2 could be introduced to approximately 1.5 billion people in these regions by substituting Golden Rice 2 varieties for conventional rice. More information on VAD is in Appendix 1.

5.3 Crop Improvement and Molecular Characteristics

5.3.1 Breeding

No rice cultivars produce carotenoids in the endosperm, but they are capable of producing the precursor, geranylgeranyl diphosphate. Phytoene synthase converts this compound to phytoene, the immediate precursor of β-carotene in plants, and

Table 5-1—Theoretical effectiveness of Golden Rice as a dietary source of vitamin A.

	Pessimistic scenario			Optimistic scenario		
	Children	Pregnant women	Lactating women	Children	Pregnant women	Lactating women
Rice intake (g/d)[a]	121.00	245.00	274.00	121.00	245.00	274.00
Current VA supply from all food sources (μg/d)[a]	234.30	597.10	404.10	234.30	597.10	404.10
RDA for VA (μg/d)[b]	500.00	800.00	1250.00	500.00	800.00	1250.00
VA deficit (μg/d)	265.70	202.90	845.90	265.70	202.90	845.90
Beta-carotene intake through GR (μg/d)	145.20	294.00	328.80	363.00	735.00	822.00
VA from GR after bioconversion (μg)	12.10	24.50	27.40	60.50	122.50	137.00
Improved VA supply (μg/d)	246.40	621.60	431.50	294.80	719.60	541.10
Contribution of GR to reduce VA deficit (%)	4.55	12.07	3.24	22.77	60.37	16.20
Efficacy (%)	11.54	24.64	9.65	47.97	86.08	40.30

[a]These figures are based on FNRI (1993). [b]These figures represent internationally used RDAs (IOM 2002).
From Zimmerman and Qaim (2004).

requires phytoene desaturase, ζ-carotene desaturase, and lycopene β-cyclase. Phytoene desaturase and ζ-carotene desaturase are involved in catalyzing the introduction of 2 double bonds, while lycopene β-cyclase is encoded by the lcy β gene. In Golden Rice 1, the former 2 enzymes were replaced by a bacterial carotene desaturase capable of introducing all 4 double bonds. Golden Rice 2 was transformed with homologues of the same genes that were used in Golden Rice 1, and encode enzymes with similar activities. The gene encoding phytoene synthase in Golden Rice 2 was isolated from maize, while the synthase gene used in the construction of Golden Rice 1 came from daffodil (*Narcissus pseudonareissus*; Paine and others 2005).

The genetic elements present in the Golden Rice 2 construct are shown in Figure 5-1 (Paine and others 2005). *Agrobacterium tumefaciens*-mediated transformation was used to introduce the DNA construct into *Oryza sativa*. The selectable marker used for transformation was the *E. coli* phosphomannose isomerase selection (PMI) system (Negrotto and others 2000). A polymerase chain reaction-based (PCR) method was used for preliminary identification of events with single copies of the transferred DNA. No additional molecular characterization of the transformants was reported (Paine and others 2005).

5.3.2 Metabolic pathway for *de novo* carotenoid biosynthesis

Carotenoid biosynthesis is well described at the biochemical and molecular level (see Appendix 2). Figure A1-2 provides a schematic overview of the pathway leading to biosynthesis of β-carotene and products derived from branches of the same pathway. Carotenoids are one of many end products of isoprenoid metabolism; however, they constitute a very small percentage of the total cellular content (for example, approximately 20 to 40 μg/g dry weight, approximately 0.0002% to 0.0004% of dry weight) in Golden Rice 2, and it is thus likely that very little metabolic energy and only small amounts of precursors are invested in their biosynthesis. Although the DNA construct present in Golden Rice 2 would be expected to have little effect on overall composition, it might be expected to alter the distribution of metabolites that are ultimately derived from geranylgeranyldiphosphate (GGPP).

5.3.3 Possible undesirable effects

The most likely undesirable effect of the modification present in Golden Rice 2 is a redistribution of metabolites that arise from GGPP, such as within the carotenoid pool or among carotenoids, tocopherols, and terpenoids (Misawa and others 1994). Perturbations in isoprenoid metabolite concentrations subsequent to pathway engineering have been reported (Misawa and others 1994). Such changes would have negligible nutritional impact on humans because rice is not a good source of any required nutrients that are formed from GGPP; however, they may have unintended effects on the physiology or metabolism of the plant itself. These changes could, in turn, indirectly affect the overall composition and nutritional value. In this regard, it is encouraging that the phenotype of the transgenic rice plants seems to be indistinguishable from that of conventional counterparts (Paine and others 2005). Keeping in mind that all plant breeding can lead to unintended changes (Cellini and others 2004), compositional analysis can reveal whether significant and meaningful changes have occurred during gene transfer and subsequent breeding.

5.4 Stage of Product Development

Golden Rice 2 represents a significant enhancement of the concept demonstrated by the construction of Golden Rice 1; namely, that grains can be engineered to contain higher levels of vitamin A precursors (see Figure 5-2). The project has resulted in the production of rice plants that produce elevated levels of total carotenoids (10 to 40 μg/g dry weight) compared to conventional rice or Golden Rice 1, of which approximately 80% is present as β-carotene. The other 20% has been characterized as β-carotene and nonprovitamin-A carotenoids. The food safety assessment of Golden Rice 1 is in progress (see http://www.goldenrice.org) and the initial transformation events are being

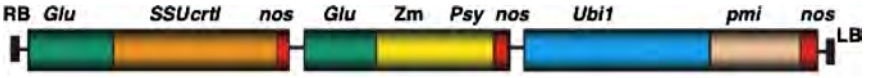

Figure 5-1—DNA construct present in Golden Rice 2 (Paine and others 2005). Key to source of DNA elements: *Glu*: rice glutelin Glut01 (Glu) promoter (nucleotides 1568--2406) *SSUcrtI:* functional fusion of the pea RUBISCO small subunit plastid transit peptide with *Erwinia uredovora crtI* (D90087; Misawa and others 1993) Terminator regions of A. tumefaciens nos (nucleotides 1848–2100, V00087). *Zea mays* phytoene synthase (psy) *Zea Mays* polyubiquitin Ubi–1 promoter with intron *E. coli* phospho-mannose isomerase (PMI) selectable marker.

Figure 5-2—Comparison of rice (left), Golden Rice 1 (middle), and Golden Rice 2 (right). (Paine and others 2005; used with permission).

introgressed into cultivars that are used in South and Southeast Asia so that agronomic and environmental impacts can be assessed. The status of Golden Rice 2 is uncertain at this time; no further reports have appeared since the initial publication (Paine and others 2005).

5.5 Safety Assessment of Golden Rice 2

The prospective food safety assessment proposed subsequently is based on the principles of scientific risk assessment of novel foods as described by various international organizations (Codex 2003; ILSI 2004). In this assessment, the principles of risk-based analysis are used to identify hazards and quantify and characterize risks. Plant breeders frequently alter the genetic composition of crops through directed selection of mutations. Hundreds of rice varieties are cultivated around the world. Although new varieties may have altered agronomic properties and compositions, the products of conventional plant breeding can be put into agricultural production without regulatory review. This is because conventional breeding is typically considered to be a safe process.

Scientific risk assessment supports the conclusion that newer methods of modifying crops using the techniques of gene transfer are no more likely than conventional breeding to produce unintended outcomes (Cellini and others 2004). The safety assessment should, thus, be focused on the safety of the crop itself and not the technology used in its breeding. Changes can be identified as differences between the content range of components observed for conventional rice varieties and the new variety; that is to say, conventional rice is a suitable comparator for Golden Rice 2. The principal questions to be answered are therefore: (1) Are the newly produced proteins safe to consume? (2) Are the newly elevated levels of carotenoids safe to consume? (3) Have there been unintended changes in composition? and (4) Have other changes in phenotype occurred, and if so, do they have any relevance to safety? (ILSI 2004).

5.5.1 History of safe use

Rice has a long history of safe use, as does β-carotene from food. Selection was performed using the phosphomannose isomerase (PMI) selection (Negrotto and others 2000). The U.S. EPA has affirmed the safety of the PMI marker system by granting an exemption to a requirement for a tolerance to be set (EPA 2004). The *crtI* gene isolated from *Erwinia uredovora* has also been used to confer herbicide resistance to rice and other plants (Misawa and others 1993, 1994). Resistance to bleaching herbicides occurs because the product of *crtI* is insensitive to the herbicides and, thus, bypasses the inhibition of carotenoid biosynthesis that leads to bleaching. The constructs used to produce herbicide tolerance in this manner encode a transit peptide and a strong promoter of high-level expression in green tissues. The constructs are, thus, quite dissimilar to those described for Golden Rice 2 and are not relevant to the present case study.

Several of the DNA elements incorporated into the construction were isolated from crop plants that also have a long history of safe use (that is, maize *psy* and Ubi–1 promoter with intron, and rice *Glu*). The *nos* terminator from *Agrobacterium tumefaciens* is frequently used in commercialized transgenic plants, while the *Erwinia uredovora crtI* and the *pmi* from *E. coli* do not have a long history of safe use in food per se, although humans certainly ingest bacteria and foods that contain homologues of these enzymes. Furthermore, *pmi* is found in strains of gastrointestinal bacteria that normally inhabit the human gut (Privalle 2003). The 3 biosynthetic enzymes, or their close homologues, expressed in Golden Rice 2 are commonly consumed in the human diet. Humans and animals consume many plant foods that are rich in mixed carotenoids. Carotenoid-containing plants also contain the enzymes of the same biosynthetic pathway that has been enhanced in Golden Rice 2. It is therefore reasonable to conclude that the introduced gene products, with the exception of carotene desaturase, have a long prior history of safe use in human nutrition. The carotene desaturase gene (*crtI*) from *E. uredovora* is almost certainly present in plant material consumed by humans and animals; this prior level of exposure is, however, uncharacterized to date.

The presence of increased levels of carotenoids in the rice endosperm is evidence that the introduced enzymes are active; however, the enzymes have not been directly characterized. Sequence comparisons with DNA and protein databases demonstrate that the inserted proteins bear no significant similarity to known toxic proteins or allergens (Codex 2003; Goodman and Wise 2006). Rice has low protein content (approximately 7.5%). The enzymes encoded by the inserted DNA are most likely expressed at very low levels. The level of expression of carotene desaturase and PMI in the grain should be determined, because that is the portion of the plant that humans consume for which dietary exposure should be estimated. The similarity of these 2 enzymes to those isolated from the donor organism should be established, particularly with regard to the potential presence of posttranslational modifications.

Rice is cooked before consumption. Thermal processing (for example, steaming of rice) denatures most proteins. It may be informative to determine the effect of processing on carotene desaturase; however, this information is not essential to the safety assessment. The great majority of dietary proteins are digested rapidly in the stomach. Gastric digestibility can be evaluated in an *in vitro* model (Thomas and others 2004). The *in vitro* digestibility of carotene desaturase should be evaluated. In simulated gastric fluid, PMI has been shown to be heat-labile and rapidly digestible (Privalle 2002). If digestible proteins are present at low levels in the consumed tissue and they bear no sequence similarity to known allergens, antinutrients, or toxicants, they can be considered safe to consume (Lehrer and Bannon 2005). The great majority of dietary proteins are not toxic, and almost without exception, those few that are display acute toxicity (Sjoblad and others 1992). To address this issue, the lack of acute toxicity of PMI was demonstrated in animal studies (Privalle 2002). Acute toxicity studies with carotene desaturase have not been reported to date. Because the vast majority of proteins consumed by humans are not toxic, the enzyme from maize has no relationship to known toxicants or allergens, and the protein is likely to be present at exceedingly low concentrations in rice, it is reasonable to conclude that the enzyme is reasonably safe without need for further experimentation. If the protein is readily digested in simulated gastric fluid, it is worth asking if an oral acute toxicity study will add materially to the overall safety assessment. Animal studies are time-consuming, expensive, and of questionable ethical practice when applied to a protein that has the characteristics just described.

Because rice allergy is uncommon, rice is classified as a non-allergenic food (Lehrer and Bannon 2005). Nevertheless, patients with cereal allergies, atopic dermatitis, and asthma display a higher frequency of rice allergy than people without these conditions (Lewis and Imber 1975; Tsai and others 1990). The major rice protein allergens have been characterized and hypoallergenic rice strains have been developed (Nakamura and Matsuda 1996; Tada and others 1996).

5.5.2 Compositional changes

Golden Rice 2 varieties contain 10 to 40 μg/g of carotenoids (Paine and others 2005); this represents a negligible change in the overall composition. The carotenoid pathway is a peripheral biosynthetic pathway. It is anticipated, but not demonstrated, that the inserted genes will have negligible effects on regulatory or biosynthetic pathways in rice and should not markedly affect overall composition. It is therefore recommended that the compositions (with respect to OECD consensus documents; OECD 2004) of Golden Rice 2 cultivars be compared with those of the isogenic strains from which they are derived. The safety and nutritional implications of any significant differences in composition should be further evaluated.

5.5.3 Metabolism of the target nutrient and potential for toxicity

In humans, β-carotene undergoes enzymatic conversion *in vivo* to retinal, which is reduced to retinol or vitamin A. Very high intake levels of vitamin A are toxic and teratogenic. Hypervitaminosis A has been reported at chronic intake levels of vitamin A that are only 10-fold higher than the RDA. In contrast, β-carotene is typically accepted as being safe because it is not toxic per se and is converted in *vivo* into retinol based on need. Excessively high levels of β-carotene can, however, lead to yellow pigmentation of the skin (Bender 2003). Golden Rice 2 varieties contain lower amounts of carotenoids and their corresponding biosynthetic elements than other foods that are commonly and safely eaten (for example, sweet potato, carrot, maize) (Holden and others 1999).

In recent years, 2 observations have shed new light on the long-held belief that β-carotene is safe to consume by all individuals (Leo and Lieber 1999). Intervention studies, designed to evaluate the potential protective effect of a β-carotene supplement against various types of cancer and heart disease based on its effectiveness as an antioxidant, yielded the unexpected result that an increased incidence of lung cancer was observed in persons who smoked heavily and consumed supplemental β-carotene (30 mg/d). Secondly, it has been demonstrated that β-carotene interacts with dietary ethanol to exacerbate, rather than ameliorate, the effects of alcohol-induced vitamin A deficiency (Leo and Lieber 1999). Thus, it appears prudent until further research is completed to avoid excessively high intakes (for example, > 10 mg/d) of β-carotene until an acceptable daily intake or tolerable upper intake level has been set. The expected levels of approximately 31 μg β-carotene per gram fresh weight of Golden Rice 2 are orders of magnitude lower than the dietary supplement levels and, therefore, are not of toxicological concern.

5.5.4 Unintended changes

As noted previously, unintended changes are a common occurrence in conventional plant breeding (Cellini and others 2004). Composition analysis, according to OECD consensus documents, should provide evidence of the presence or absence of unintended changes. The safety and nutritional significance of those changes can then be characterized. Change per se is not necessarily undesirable. The phenotypic and agronomic properties of selected cultivars are also powerful indicators of the presence or absence of unintended changes; in the case of Golden Rice 2, preliminary observation suggests that no changes have occurred (Paine and others 2005). The changes introduced into Golden Rice 2 are peripheral to central metabolism and should divert very little cellular energy and metabolites to biosynthesis.

5.6 Nutritional Assessment

Cooked polished rice is essentially free of known antinutrients; most of the antinutrients are removed with the bran and/or are destroyed by heat (OECD 2004). The recommended list of relevant analyses for food uses of polished rice is proximate analysis, minerals, vitamins, total amino acids, total fatty acids, phytic phosphorus, and amylose (OECD 2004).

Golden Rice 2, which is deep orange in color, was developed to greatly increase the β-carotene of rice (Paine and others 2005). It contains as much as 37 μg total carotenoids/g dry weight of grain, of which 31 μg/g is β-carotene. Due to the higher level of β-carotene, the consumption of Golden Rice 2 in typical quantities may provide adequate daily intake of vitamin A in countries in which rice is a staple food, assuming cooking losses of β-carotene are not excessive. If proven effective, Golden Rice 2 could provide adequate levels of vitamin A to large populations that are currently at risk for VAD. Golden Rice 2 may also be effective even in populations where rice is a smaller dietary component. Although not safety related, stability, bioefficacy, and consumer acceptability will need to be established as a practical matter.

As noted previously, a significant portion of global VAD occurs in populations that consume rice as a major staple food. Substitution of Golden Rice 2 for conventional rice may eliminate a significant portion of VAD. In fact, since a typical serving of 60 to 75 g of Golden Rice 2 can conservatively be estimated to provide about half of the recommended daily intake of vitamin A for an adult male, Golden Rice 2 could be introduced as a dietary supplement rather than a direct total rice replacement. This might reduce concerns about consumer acceptability in cultures that highly value white rice as a traditional food, such as South and Southeast Asia. Attitudinal survey studies performed in 2 rice-growing villages in the Philippines indicated that yellow color was of minor concern to potential consumers of Golden Rice 1, while taste and cost were important (Zimmerman and Qaim 2004). Because it is necessary to consume only 50 to 100 g or 700 to 1400 kJ per day ($^1/_4$ to $^1/_2$ cup) of Golden Rice 2, it represents a viable dietary approach to reducing VAD anywhere in the world where this portion of rice can be supplied. Although stability of storage and cooking is as yet undocumented, preliminary results (2007 personal communication from R Russell, unreferenced) suggest that β-carotene in Golden Rice is highly bioavailable.

To be converted to nutritionally useful vitamin A, β-carotene must be absorbed and converted to retinol. The bioavailability of β-carotene varies with the species of carotenoid, molecular linkage, amount of carotenoids in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, and interactions (Castenmiller and West 1998). An extensive discussion on bioavailability is included in Appendix 2. It has been suggested (Yeum and Russell 2002) that β-carotene may be better absorbed from rice than from green leafy vegetables because of its higher digestibility (Zimmerman and Qaim 2004). It is also known that β-carotene absorption is decreased in participants whose diet is extremely low in lipid, although Jayarajan and others (1980) have shown that as little as 5 g of fat per meal is all that is needed for carotenoid absorption. Estimates of the vitamin A bioavailability from β-carotene delivered in a rice matrix vary from a best-case estimate of 1:2 to a worst-case estimate of 1:12 (Yeum and Russell 2002). The 12:1 conversion ratio of β-carotene to vitamin A is generally used as a representative uptake from plant materials, although there is considerable variation in observed values (IOM 2001). As noted previously, preliminary data indicate that a conversion factor of 4:1 may be more appropriate (2007 personal communication from Russell, unreferenced).

Based on published data, it can be estimated that to meet their vitamin A requirements, a breastfed child and nonbreastfed child during the 2nd year of life need an extra 1.92 to 2.4 mg and 3.6 mg of β-carotene a day, respectively (Hess and others 2005). Using Thai food intake data, it can be calculated that 1- to 2-y-old breastfed children eating 160 g (standard deviation 29 g) of Golden Rice 2 per day (which equates to approximately 67 g uncooked rice providing approximately 2.1 mg β-carotene would consume approximately 5 times their RDA. This is a promising result because consumption of 65 to 70 g would prevent most of the negative effects of VAD; however, the calculation assumes a 12:1 conversion ratio for β-carotene to vitamin A. If the conversion is more efficient, even more vitamin A will be available, and even if it is less efficient, Golden Rice 2 could still be beneficial because absorption and/or bioconversion is better among individuals with low vitamin A status (de Pee and West 1996; Castenmiller and West 1998). These 2 alternatives underscore the need for human bioavailability and efficacy to be assessed as soon as it is practicable.

Beta-carotene in fresh plant materials undergoes postharvest degradation, especially when exposed to light and air. Degradation and postharvest losses are less likely to occur in the dry rice matrix (Zimmerman and Qaim 2004); however, this must be tested. Heating at elevated temperature can slowly degrade β-carotene, but little degradation should occur at the temperatures at which rice is steamed (Dietz and others 1988). No change in preparation or use will be required for the introduction of Golden Rice 2.

5.7 Conclusions

Vitamin A deficiency is a major public health challenge. The need and applications for Golden Rice 2 are identical to those for Golden Rice 1. Golden Rice 2 is formally analogous to Golden Rice 1; however, a gene isolated from daffodil has been replaced by a more efficient gene analogue isolated from maize. When compared to Golden Rice 1, the new rice variety represents a significant enhancement of total carotenoid production in rice, with β-carotene being the predominant form.

Golden Rice 2 is in an early stage of development, establishing the proof-of-concept. The relevant genes are now being crossed into selected local cultivars in several Asian countries to allow further evaluation of agronomic efficacy and safety studies (see http://www.goldenrice.org/ for the latest information). The safety considerations that apply to Golden Rice 1 and 2 are similar. The components found in Golden Rice 2 have a history of safe use. No novel proteins or metabolites not normally encountered in the human diet are known to be present; any novel proteins present are at exceedingly low concentrations. The safety of carotene desaturase stands out as the single component that warrants further characterization. It is recommended that the molecular form of the protein and its *in vitro* digestibility be analyzed, but it is considered unnecessary to assess its acute toxicity in animal studies if it is digestible—such studies would add little additional assurance of safety. The concentration of carotenoids is similar to that encountered in other commonly eaten foods. Rice allergy is not common and rice is not a significant source of toxicants or antinutrients. If no undesirable or adverse compositional changes are encountered upon analysis, Golden Rice 2 will present insignificant food safety risks.

Effective risk management is based on the premise that the stringency of analysis should be commensurate with risk, and the degree of risk that is acceptable must be weighed against the benefits of approving a new crop variety along with the consequences of nonadoption. In the case of Golden Rice 2, the most significant concern is whether it will alleviate VAD as intended. Ultimately, it is the role of risk managers to determine a priori what balance of risk and benefits are acceptable (FAO/WHO 1997). Al-Babili and Beyer (2005) (see Appendix 5), however, have argued strongly that Golden Rice should not become mired in a regulatory review that seeks to evaluate and eliminate every conceivable hazard.

Though not directly related to safety, there are matters of practical concern that should be addressed as part of a comprehensive release strategy. While theoretically capable of supplying nutritionally relevant levels of β-carotene, the retention of β-carotene after processing, storage, and cooking needs to be determined before efficacy is demonstrated. Moreover, some have questioned if yellow rice will prove acceptable to people who traditionally eat white polished rice. All of these points need to be resolved if Golden Rice 2 is to become an important solution to VAD.

5.8 Recommendations

Recommendation 1. A safety assessment needs to be undertaken. Specific analysis could include:

- Characterization of the DNA insert
- Characterization of the inserted proteins (posttranslational modification)
- Digestibility (*in vitro*) of carotene desaturase
- Composition analysis
- Carotenoid metabolite pool analysis

Recommendation 2. In addition to the safety studies, the prediction that Golden Rice 2 could greatly improve vitamin A

nutrition should be tested in premarket studies in free-living humans, including palatability of rice with altered appearance to conventional rice.

Recommendation 3. Any potential risks that may be identified with Golden Rice 2 should be balanced against the potential to ameliorate VAD and, thereby, reduce the loss of life and clinical symptoms due to the nutritional deficiency. The magnitude of this potential nutritional impact will only become known for certain if Golden Rice 2 is adopted.

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Chapter 6: Maize with Increased Lysine (Lysine Maize—LY038)

ABSTRACT: Data and information provided in this case study relate to a crop derived by modern biotechnology, in which a specific nutrient (lysine) has been increased in maize grain. Lysine maize is a feed ingredient with enhanced nutritional characteristics for poultry and swine and provides an alternative to adding supplemental lysine to diets for these animals. Lysine maize is in an advanced state of development; therefore, extensive unpublished data and information are presented to demonstrate that (1) Lysine maize, and the feeds and foods derived from it, are as safe as those derived from conventional maize, and (2) the increased lysine in Lysine maize grain produces the intended nutritional benefit for broiler chickens when compared to a diet containing conventional maize grain and a crystalline lysine supplement. These conclusions are based on a detailed molecular characterization of Lysine maize, a safety assessment of the introduced protein, a safety and nutritional assessment of the LY038 crop, and a comparison of the agronomic and phenotypic properties of maize hybrids with and without the Lysine maize trait. Although Lysine maize is a specialty crop for use in animal feed, its safety for both animals and humans must be demonstrated. Free lysine is significantly increased in Lysine maize by the introduction of the *dapA* gene (*cordapA*) from *Corynebacterium glutamicum* that encodes a form of dihydrodipicolinate synthase (cDHDPS) that is insensitive to lysine feedback inhibition. Analysis of lysine anabolic and catabolic pathways in maize identified 6 metabolites that might change as a consequence of the introduction of cDHDPS insensitive to lysine-feedback inhibition. The results of compositional analysis demonstrated that Lysine maize grain is comparable to conventional maize, with the exception of the intended increase in lysine and a corresponding increase in 2 products of lysine catabolism, saccharopine and α-aminoadipic acid. Therefore, the safety and/or nutritional implication of these 3 compounds under the conditions of use were the focus of additional assessments and found to not present either a safety or nutritional problem.

6.1 Introduction

Animal production is often restricted because feed resources are deficient in a specific nutrient or because the bioavailability of a nutrient is low or is constrained by the presence of an antinutritional factor. Several crops have been developed and are currently in trials with modifications aimed at improving nutritional characteristics in which the concentration of a specific nutrient, such as an essential amino acid, is increased. This case study examines the safety and nutritional assessment of maize grain in which modern biotechnology has been used to increase the nutritional value by increasing the lysine content of the grain.

6.1.1. The need for the enhanced nutrient

Poultry and swine diets based on maize and soybean meal require supplemental lysine for optimal animal growth and production (NRC 1994, 1998). Supplemental L-lysine is commercially available and is typically produced via fermentation of *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* (Eggeling 1994). As an alternative to supplementation, conventional- and biotechnology-based strategies have been developed. Conventional breeding research resulted in the development of Quality Protein Maize (QPM), which contains twice the normal amount of lysine (CiMMYT). Lysine maize (LY038) was developed through the use of recombinant DNA techniques to integrate the *cordapA* gene into the maize genome, which results in the production of maize grain with higher lysine content. This grain with enhanced lysine concentration has an improved nutritional value (similar to QPM) for use as a feed ingredient in diets for broiler chickens, turkeys, and pigs.

6.2 The Crop and Its Role in Nutrition

6.2.1 The maize crop

Maize (*Zea mays* L.) is an annual, wind-pollinated, monoecious species belonging to the subfamily Panicoideae of the grass family Gramineae. It was probably domesticated in the highlands of southern Mexico and grown as a food crop as early as 2700 BC. By the time Columbus arrived in the New World, maize was being grown by the indigenous civilizations from Chile to southern Canada. When on the north coast of Cuba, Columbus noted in his log (November 6, 1492) that crew members reported "a sort of grain called maize which was well tasted, baked, dried and made into flour." Within 2 generations of the introduction of maize into the Old World by Columbus in 1494, it had spread as far east as China and was widely grown in many areas of the world.

6.2.2 How the maize crop is used for food and feed

Today, maize ranks third after wheat and rice as one of the world's 3 leading food grains, is grown on 140 million ha in 100 countries, and produced 700 million metric tons of grain in 2004. The major producers of maize are the United States, China, Brazil, Mexico, France, and India and account for 75% of world production (FAOSTAT 2004). However, unlike wheat and rice, the majority of maize grain produced in the northern hemisphere is fed to livestock, whereas maize is a major staple food for humans in the tropics and the southern hemisphere.

While maize grain is often the preferred dietary energy source for both ruminant and nonruminant livestock production systems, it is recognized that its low lysine content requires

supplementation to optimize animal performance in pigs and poultry.

6.2.3 Expected geographic distribution of the nutritionally enhanced crop

Lysine maize will be a value-added specialty crop for use as an animal feed ingredient. Lysine maize is expected to be used to replace both the crystalline lysine and conventional maize in many diets that are already nutritionally balanced for optimal animal growth performance. It is anticipated that Lysine maize will be produced in the United States and Argentina, with use of the grain in both domestic and export markets.

6.2.4 Expected dietary impact and bioavailability

Lysine maize will simplify diet preparation by providing an alternative to direct addition of supplemental lysine to poultry and swine diets by increasing the amount of free lysine in the grain. Furthermore, its production is faster than the development of QPM via conventional breeding techniques. The lysine content of conventional maize grain ranges from 2500 to 2800 mg/kg on a dry matter (DM) basis and is largely incorporated into storage proteins (free lysine is approximately 40 mg/kg). By comparison, the total lysine in Lysine maize grain is 3400 to 5200 mg/kg on a DM basis. This increased level of lysine is due to an increased content of free lysine (approximately 1500 mg/kg).

Production and processing of feed from Lysine maize are not expected to differ from those of conventional maize. To preserve its enhanced nutritional value, appropriate commercial practices are needed to minimize grain loss between harvesting and livestock producers. However, even if Lysine maize were inadvertently used in human food, it would be considered safe because lysine is recognized as safe and the levels of lysine and related metabolites would be similar to or lower than those present in foods with a history of safe consumption. Thus the presence of adventitious Lysine maize in the market should have no health or nutritional effects different from those of conventionally derived high lysine maize (QPM) already in commercial use.

6.3 Crop Improvement and Molecular Characterization

6.3.1 Crop transformation and breeding

To produce Lysine maize, a linear piece of DNA from a plasmid vector containing the *cordapA* and *nptII* coding sequences was introduced into the maize inbred line H99. The *nptII* gene encodes resistance to a category of aminoglycosides including kanamycin, neomycin, and paromomycin. When cultured in the presence of neomycin, only successfully transformed plant cells continued to grow. Plants regenerated from these cells were assayed for the presence of the *cordapA* gene by polymerase chain reaction (PCR) and only positive plants continued being propagated. A diagram showing the insertion of the LY038 insert and *nptII* excision by cre/lox is presented in Figure 6-1. The *nptII* cassette was flanked by 2 *lox*P sites to allow for its subsequent

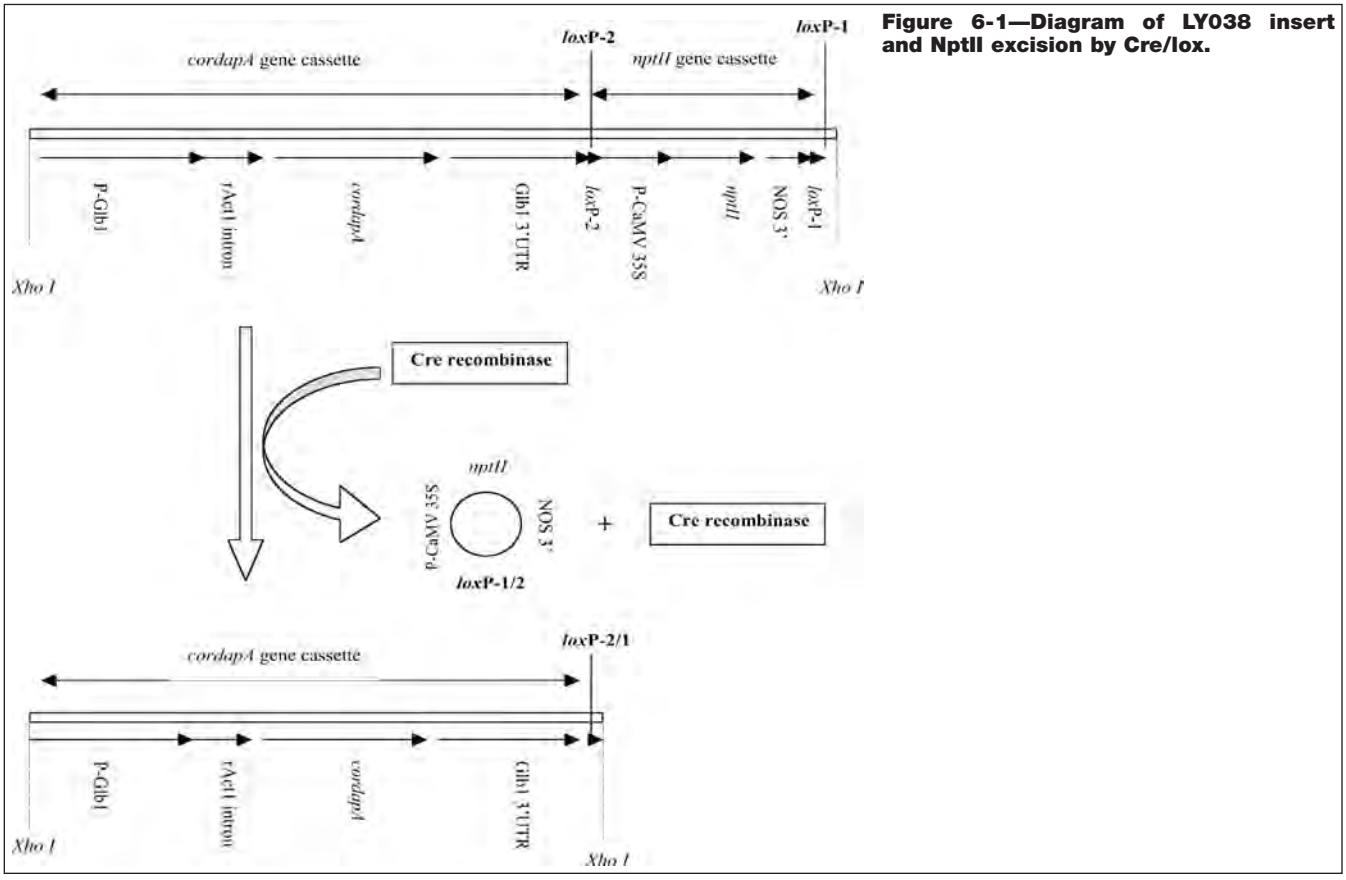

Figure 6-1—Diagram of LY038 insert and NptII excision by Cre/lox.

removal by Cre recombinase (Gilbertson 2003). An extensive number of Southern blots, including generational stability analyses over multiple generations, established that there were no genetic elements from the *cre* and *nptII* coding sequences detected in Lysine maize.

The *cordapA* gene encodes a dihydrodipicolinate synthase from *Corynebacterium glutamicum* (cDHDPS). The *cordapA* gene is controlled by the *Zea mays* globulin 1 (Glb1) promoter that directs cDHDPS protein expression and subsequent accumulation of free lysine to the maize germ. The intron sequence following the Glb1 promoter is derived from the rice actin-1 gene and its purpose is to enhance DNA transcription (McElroy and others 1990). Because cDHDPS is a bacterial enzyme, the *cordapA* coding sequence is preceded by the *Zea mays* dihydrodipicolinate synthase chloroplast transit peptide (mDHDPS CTP). The function of the mDHDPS chloroplast transit peptide is to translocate cDHDPS to the plastid, where lysine biosynthesis occurs via the aspartate pathway (Frisch and others 1991).

The *cordapA* gene is from *Corynebacterium glutamicum*, a common soil microorganism that has been used for decades in the industrial production of lysine (Eggeling and others 1998). Dihydrodipicolinate synthase (DHDPS) is the first and major rate-limiting enzyme for lysine biosynthesis in plants and bacteria (Azevedo and Lea 2001) and is regulated by lysine feedback inhibition (Galili 1995). However, compared to the DHDPS in maize, this enzyme from *C. glutamicum* (cDHDPS) is relatively insensitive to lysine inhibition (Vauterin and others 2000). The LY038 plants expressing the cDHDPS protein are under the control of the *Zea mays* globulin 1 (Glb1) promoter and accumulate-free lysine in the grain.

Analyses showed that LY038 contains 1 intact copy of the *cordapA* gene cassette inserted at a single site in the maize genome. Analysis of the genomic DNA flanking the inserted DNA shows no concerns. No additional elements from the transformation vector, linked or unlinked to the intact gene cassette, were detected in LY038. LY038 does not contain either intact or partial DNA fragments of the *nptII* cassette or the *cre* cassette and also lacks detectable backbone sequence from the transformation plasmids. The presence of the *cordapA* gene cassette and the absence of both *cre* and *nptII* gene cassettes in LY038 were further confirmed by Southern blot generational stability analyses over multiple generations representing each branch point of the LY038 breeding tree. Analyses by PCR confirmed the organization of the genetic elements of the inserted DNA in LY038 to be identical to that in the transformation plasmid.

6.3.2 Possible undesirable effects

To be commercially successful, all crops derived from modern biotechnology undergo extensive breeding with elite lines such that > 90% of the germplasm of commercialized lines will be derived from elite lines that have not experienced genetic transformation. This should significantly reduce opportunities for transformation-induced, random changes in the genome being in the final product. Replicated field trials in multiple locations in Argentina and the United States were conducted to assess whether genetic modification resulted in unintentional phenotypic and agronomic changes in Lysine maize compared with conventional hybrids. Phenotypic characteristics (for example, seedling vigor, early stand count, days to 50% pollen shed, days to 50% silking, stay green, ear height, plant height, dropped ears, stalk lodged plants, root lodged plants, final stand count, grain moisture, test weight, and yield) were statistically evaluated and compared among Lysine maize, a control (near isogenic counterpart), and reference maize hybrids within each field site and across all field sites for a given geographical zone. The results showed that the genetic modifications to generate Lysine maize did not unintentionally alter the phenotype of Lysine maize plants. Furthermore, dormancy and germination characteristics of Lysine maize were unaltered compared to its control and no differences in pollen characteristics, more specifically pollen morphology and viability, were detected when comparing Lysine maize to its control. It is, therefore, possible to conclude that Lysine maize has shown similar agronomic behavior and phenotypic properties to conventional maize.

6.3.3. Metabolic pathways

In GM crops with improved nutritional characteristics, metabolic pathways are often modified to achieve the desired nutritional improvement, and a thorough understanding of the changes that have occurred is important for both the safety and nutritional evaluation of the GM crop. The metabolic pathways altered in the development of Lysine maize are well characterized and are shown in Figure 6-2 for lysine anabolism and in Figure 6-3 for lysine catabolism in maize. Based on the analysis of these pathways, the lysine-related metabolites described in section 6.5.2 were selected as specific targets for compositional analysis.

6.3.4. Stage of product development

The development of Lysine maize is well advanced and a full safety and nutritional assessment has been conducted.

6.4 Safety Assessment

6.4.1 History of safe use

It is well recognized that absolute safety is not an achievable goal in any human endeavor, and this is relevant with respect to food and feed. The safe use of food or feed has typically been established either through experience based on its common use or by application of generally recognized scientific assessment procedures. Since the 1990s, the standard for novel (especially GM) food and feed crops has been that they should be as safe as an appropriate counterpart with a history of safe use. This comparative assessment process identifies similarities and differences between the newly developed food or feed crop and a conventional counterpart with a history of safe use. The similarities noted between the new and traditional crops are not subject to further assessment because this provides evidence that those aspects of the newly developed crop are as safe as crops with a history of safe consumption. The identified differences are subjected to further scientific procedures, as needed, to clarify whether any safety issues or concerns exist.

6.4.2 Compositional changes

Compositional analysis is considered the cornerstone for the safety and nutritional evaluation of GM crops. In the current case

study, extensive compositional analyses of forage (whole plant at early-dent stage) and grain have been performed on samples from replicated, multisite field trials conducted in both Argentina (2001 to 2002) and the United States (2002) to compare the composition of Lysine maize to its control (a near isogenic counterpart) and conventional maize. Lysine maize forage samples were subjected to compositional analyses for proximates (protein, fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), lysine, and minerals (calcium, phosphorus), as well as carbohydrates by calculation. Compositional analyses of Lysine maize grain samples included proximates (protein, fat, ash, moisture), ADF, NDF, total dietary fiber (TDF), amino acids, free lysine, fatty acids (C8–C22), vitamins (thiamin, riboflavin, B6, E, niacin, and folic acid), antinutrients (phytic acid and raffinose), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), carbohydrates by calculation, secondary maize metabolites per OECD (2003) consensus document (furfural, ferulic acid, and ρ-coumaric acid), and additional lysine-related metabolites (cadaverine, α-aminoadipic acid, saccharopine, homoserine, L-pipecolic acid, and 2,6-diaminopimelic acid). Homoserine and 2,6-diaminopimelic acid were chosen from the lysine biosynthetic pathway because they are stable metabolites and constitute either a key branch point or the penultimate synthetic step for making lysine, respectively. Cadaverine, saccharopine, α-aminoadipic acid, and pipecolic acid were included because they represent the stable components of lysine

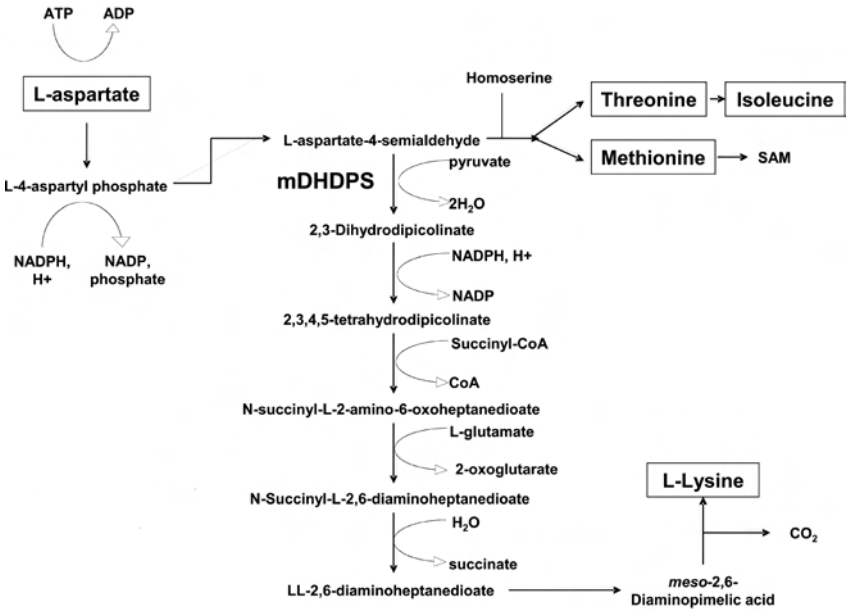

Figure 6-2—Lysine anabolism in maize.

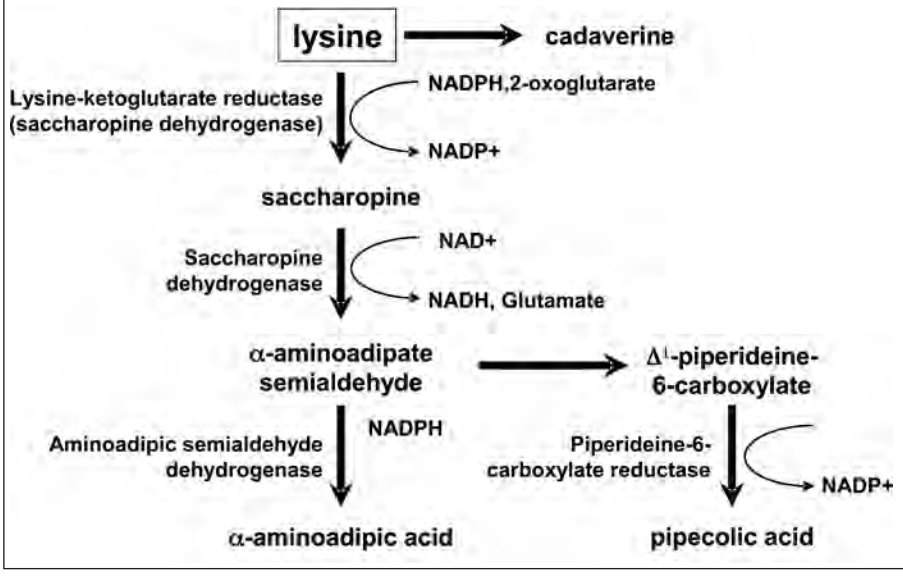

Figure 6-3—Lysine catabolism in maize.

catabolism known to accumulate in plants. In all, 85 different analytical components (75 in grain, 10 in forage) were analyzed.

The compositional analyses of grain and forage of Lysine maize showed them to be compositionally equivalent to that of conventional maize, except for the intended increase in grain lysine content and an associated increase in the lysine-related catabolites, saccharopine and α-aminoadipic acid, supporting the absence of unintended effects due to the genetic modification.

6.4.3 Protein safety assessment

Both Codex Alimentarius (Codex Alimentarius 2003) and the European Food Safety Authority (EFSA 2004) have recognized the fact that a weight-of-evidence approach is appropriate for the safety assessment of novel proteins, as numerous factors contribute to whether they have the potential to be allergenic or toxic. These factors include the source of the protein, sequence homology to known allergens and/or toxins, and an assessment of protein digestibility. The digestibility data are of value only in the context of an overall "weight-of-the-evidence" assessment.

6.4.3.1 History of safe exposure to donor organism. Human consumption of the cDHDPS protein from processed grain products is expected to be low because Lysine maize grain is not intended to be used in food and because expression of cDHDPS is primarily in the germ. The endosperm is the predominant fraction humans consume. Nonetheless, several safety assessments of cDHDPS were conducted.

C. glutamicum is a common soil bacterium. Humans and animals are regularly exposed to this organism and/or its components (for example, cDHDPS) without adverse consequences. All *C. glutamicum* cultures available from the American Type Culture Collection are classified at Biosafety Level-1, the safest of all cultures as defined by the U.S. Dept. of Health and Human Services (CDC 1999). In addition, DHDPS proteins functionally and structurally related to cDHDPS in Lysine maize are present in plants and microbes that synthesize lysine, many of which are consumed as feed and/or food such as maize.

Bioinformatic analyses revealed no biologically relevant sequence similarities of the cDHDPS protein to known allergens, toxins, or pharmacologically active proteins. Furthermore, no short (8 amino acid) polypeptide matches are shared between the cDHDPS protein sequence and known protein allergens. These data demonstrated the lack of both structurally and immunologically relevant similarities between allergens or toxins and the cDHDPS protein sequence used in Lysine maize.

6.4.3.2 *In vitro* digestibility. *In vitro* digestibility and acute mouse toxicity studies with cDHDPS utilized protein produced and purified from *E. coli*. Before initiation of these studies, cDHDPS protein produced in *E. coli* was compared to cDHDPS expressed in Lysine maize by several methods, including enzymatic activity assays, determination that maize cDHDPS is not glycosylated, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and N-terminal sequence analysis. From these analyses, the cDHDPS protein purified from *E. coli* was shown to be physicochemically and functionally equivalent to cDHDPS produced in Lysine maize.

Previous studies assessed the susceptibility of proteins expressed in genetically modified plants to *in vitro* proteolytic digestion (Kimber and Dearman 2002) following a standard protocol (Pharmacopeia 1995). Recently, ILSI standardized the pepsin digestibility assay protocol in a multilaboratory evaluation (Thomas and others 2004). The *in vitro* digestibility of *E. coli*-produced cDHDPS protein was assessed in simulated gastric fluid (SGF) containing the proteolytic enzyme pepsin following a time course, and experimental parameters similar to conditions used in the ILSI multilaboratory evaluation.

The digestibility of the cDHDPS protein was evaluated by visual examination using stained SDS-PAGE gels and western blot analysis. Visual examination of the stained gel showed that the full-length (33 kDa) cDHDPS protein was rapidly digested after incubation in SGF. Based on the limit of detection (LOD) for the cDHDPS protein in SGF, it can be inferred that > 96% of the cDHDPS protein was observed to be digested in SGF within 30 s. Western blot analysis confirmed that > 98% of the cDHDPS protein was digested below the LOD of this immunoassay within 30 s of incubation in SGF. No stable peptide fragments were observed by either stained SDS-PAGE gels or by western blot analysis. Therefore, the demonstrated rapid degradation of cDHDPS in SGF, combined with the history of safe exposure to the donor organism and the lack of sequence homology to known allergens and toxins, supports a conclusion that the cDHDPS protein has low allergenic and toxic potential.

6.4.3.3 Acute high-dose oral toxicity study of cDHDPS. An acute high-dose oral toxicity study was considered appropriate to assess the safety of *E. coli*-produced cDHDPS protein. In this study, 2 groups of 10 male and 10 female mice were given a single gavage dose of 800 mg/kg body weight cDHDPS protein. Following administration of the cDHDPS protein, the mice were observed daily and weighed weekly during a 14-d period. There was no mortality and were no reports of adverse clinical reactions. All cDHDPS-dosed mice gained weight during the 14-d postdosing period. Weight gain and food consumption were comparable to those of controls. A gross necroscopy examination was conducted on all animals at study termination. The macroscopic appearance of cDHDPS-dosed mice was within normal limits for CD-1 mice and similar to the controls. No toxicity was observed in any of the groups. Therefore, the no observed effect level (NOEL) for the cDHDPS protein was determined to be greater than 800 mg/kg body weight, the highest dose tested.

6.4.3.4 Plant expression of cDHDPS in Lysine maize and dietary risk assessment. The levels of cDHDPS in grain were higher than those in other plant tissues (26, 0.081, and 0.94 μg/g dry weight in grain; whole plant at V2-V4 growth stage; and forage at the R5 growth stage, respectively) when measured by enzyme-linked immunosorbent assay (ELISA). This is consistent with the fact that *cordapA* gene expression is predominantly targeted to the germ of the grain by the Glb1 promoter in LY038. Based on cDHDPS levels in grain and the determined NOEL from the mouse acute oral toxicity evaluation, large margins of exposure were calculated for cDHDPS for livestock (> 500-fold for broiler chickens and pigs) and humans (> 45000 for the highest consuming U.S. subpopulation and > 10^7 for the highest European Union subpopulation, using conservative assumptions).

This assessment leads to the conclusion that there is no meaningful risk to animal or human health from dietary exposure to cDHDPS from Lysine maize.

6.4.3.5 Lysine safety. Lysine is an essential amino acid and is generally recognized as safe (GRAS) when added to animal diets at nutritional levels (U.S. FDA 1982) and may be safely used as a human food additive when used as a nutrient (U.S. FDA 1972). Furthermore, excessive consumption of lysine by humans, pigs, and rats over prolonged periods is well tolerated (Garlick 2004). In plants and animals, lysine is primarily catabolized via the saccharopine pathway by 2 linked enzymes, lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) (Miron and others 2000).

Because ingested lysine is largely degraded through the saccharopine pathway in humans, animals, and plants, tissues would be transiently exposed to higher than normal levels of saccharopine and α-aminoadipic acid. However, it is anticipated that farm animals consuming Lysine maize grain would readily degrade saccharopine based on the liver capacity of SDH (Fellows and Lewis 1973). Thus, the increased levels of saccharopine and α-aminoadipic acid in Lysine maize would not be expected to pose a health risk for livestock, and would not be expected to accumulate in livestock products any differently than when lysine is included as a supplement in livestock diets.

Even though Lysine maize grain will be identity preserved to facilitate recovery of its enhanced nutritional feed value, it cannot be ruled out that a small portion of the grain might inadvertently be used for human food. If this occurred, humans would experience only a short-term, limited dietary exposure to Lysine maize grain as it would be diluted by other commodity maize during harvest, transport, storage, and food processing. In addition, saccharopine and α-aminoadipic acid are measurable components of safely consumed foods, supporting a history of dietary exposure and safe consumption of these 2 metabolites by humans. Furthermore, confirmatory animal feeding studies in broilers and rats provide supporting data regarding the safety of Lysine maize grain for humans because maize grain exposure levels were orders of magnitudes higher than potential human consumption levels for corn grain-based products (see the following sections 6.4.3.6 and 6.5). Therefore, there is reasonable certainty that the levels of saccharopine and α-aminoadipic acid in Lysine maize are not harmful to either animal or human health.

6.4.3.6 A subchronic rat toxicology study of Lysine maize grain. The safety of Lysine maize grain has been further assessed by a 90-d feeding study in rats (FSANZ 2006). The study compared the responses of rats fed diets containing grain from Lysine maize at either 11% or 33% of the diet, its near isogenic control, and 6 diets with traditional maize hybrids. Lysine maize and control grain were produced at the same time and under the same environmental conditions, and traditional reference grains were purchased from commercial sources. Toxicological parameters such as survival, body weights, food consumption, clinical pathology, organ weights, and macroscopic and microscopic pathology were evaluated in this study. There were no test article-related changes in any of the toxicological parameters. No adverse effects on growth, health, or behavior were reported in rats fed Lysine maize grain at up to 33% of the diet for at least 90 d.

6.5 Nutritional Assessment

6.5.1 Lysine supplementation of broiler diets

Broiler chicken and swine diets based on maize and soybean meal may require the addition of supplemental lysine for optimal animal performance (NRC 1994, 1998). Supplemental lysine is usually in the form of lysine monohydrochloride or lysine sulfate (Leuchtenberger 1996) produced via fermentation by *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* (Eggeling 1994). Both of these lysine sources are highly bioavailable (Sibbald and Wolynetz 1985; Nelson and others 1986; Izquierdo and others 1988), and their addition to lysine-deficient diets improves the growth rate and feed efficiency of rapidly growing broiler chickens relative to birds fed similar diets without supplemental lysine (Emmert and Baker 1997). Relatively small changes in growth rate, feed efficiency, and/or carcass measurements as a result of a change in nutritional (nutrient or antinutrient) or health status can be detected in the fast growing broiler (Hammond and others 1996; Taylor and others 2003a, 2003b, 2003c).

6.5.2 Performance

Studies using day-old broiler chicks, which are recognized as being sensitive to small changes in nutritional composition, have been widely used to compare nutritional performance obtained from conventional and GM feeds with agronomic input traits (NRC 1994; Taylor and others 2003a, 2003b, 2003c). While such studies are not seen as a key component of the safety assessment, EFSA (2004) has suggested that they may provide supplemental information on the possible occurrence of unintended effects. In the case of nutritionally enhanced crops, performance studies with target species are essential to demonstrate the expected nutritional enhancement occurs. In the case of Lysine maize, a trial with fast growing broiler chickens was conducted to compare the performance (growth rate, feed efficiency, and carcass characteristics), bioefficacy, and bioavailability of lysine in Lysine maize with a conventional maize grain of comparable genetics and 4 reference maize varieties. Each of the control and reference maize diets were formulated with and without supplemental crystalline lysine so that the diets with the supplemental lysine had a similar dietary lysine concentration to the Lysine maize grain diet. Bird performance and health observations throughout the study also provided a basis for assessing whether there were any unexpected effects on broiler health and performance.

No unexpected effects on bird performance or health were observed when feeding Lysine maize grain. The bioefficacy and bioavailability of the lysine in Lysine maize grain were demonstrated by the improved performance of birds receiving a diet with Lysine maize compared to broilers fed a diet without supplemental crystalline lysine, but otherwise identical to the control and traditional reference maize varieties. Importantly, the performance and carcass measurements of birds fed diets with Lysine maize grain were comparable to those of birds fed diets supplemented with crystalline lysine and either near isogenic control or conventional reference maize at the same inclusion rate. Therefore, Lysine maize grain can be considered as safe as traditional maize when fed to poultry and more nutritious than traditional maize because of the increased lysine levels in Lysine maize.

6.6 Conclusions

The data and information in this case study provide an example of a GM crop in which a specific nutrient has been increased and which is to be used as a feed ingredient. This case study has demonstrated that, although each product must be considered on a case-by-case basis, the comparative safety assessment process successfully applied to agronomic trait GM crops is also appropriate and recommended for the safety and nutritional assessment of nutritionally enhanced crops derived through modern biotechnology. For the present case of Lysine maize, the available data show that it is as safe as conventional maize while being nutritionally enhanced for poultry diets.

For Lysine maize specifically, it is concluded that (1) Lysine maize is as safe as conventional maize; and (2) the increased lysine in Lysine maize grain produced the intended nutritional benefit for broiler chickens when compared to conventional maize grain with a crystalline lysine supplement.

These conclusions are based on several categories of assessment that included:

1. Detailed molecular characterization.

2. Comparison of the phenotypic, agronomic, and compositional properties of LY038 to conventional maize hybrids. The results of the comparative safety assessment studies demonstrated that LY038 grain is substantially equivalent to conventional maize with the exception of the intended increase in lysine and the corresponding increase in 2 lysine catabolites.

3. Safety assessment of the cDHDPS protein with respect to allergenicity and toxicity showed no major concerns.

6.7 Recommendations

Some of the possible key recommendations for the safety and nutritional assessment of an animal feed product with improved nutritional characteristics like Lysine maize are included below.

Recommendation 1. Comprehensive assessment of the safety of the introduced protein, including an understanding of the history of safe consumption by humans and animals of this protein and related proteins, a demonstration that the protein lacks homology to known toxins and allergens, a demonstration that the protein is rapidly degraded by digestive enzymes, and an acute toxicity test in animals at levels that greatly exceed any anticipated exposure from consumption of the product.

Recommendation 2. Consideration of the targeted metabolic pathway to identify metabolites in the synthesis or degradation pathways of the intended change that might accumulate, and inclusion of these in the compositional analysis. In Lysine maize, the well-understood metabolic pathways involved in lysine metabolism in plants and animals identified the addition of 7 lysine-related metabolites (free lysine, cadaverine, a-aminoadipic acid, saccharopine, homoserine, L-pipecolic acid, and 2,6-diaminopimelic acid) to the OECD list of targeted analytes for maize for the detection of alterations in the composition of grain from Lysine maize compared to conventional maize.

Recommendation 3. Studies in laboratory animals are useful in confirming the overall safety of the product established by the other components of the safety assessment, thereby providing added safety assurance.

Recommendation 4. Nutritional studies to demonstrate efficacy of the nutritional change in the target species. For Lysine maize, broiler feeding studies were useful to demonstrate the improved animal feed nutritional property associated with the increased lysine in LY038 maize grain.

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Appendix 1: Vitamin A Deficiency: A Global Risk

Vitamin A deficiency (VAD) is a global public health challenge, particularly in infants, children, and pregnant and lactating women because of the extra requirements to support fetal, infant, and child growth (Figure A1-1). Deficiency can result in reduced resistance to infection, impaired cellular differentiation, xerophthalmia, and ultimately blindness and death; it is also associated with anemia. More than 100 million children and as many as 7 million pregnant women living in over 100 countries have VAD (West 2002; WHO 2003). It is estimated that between 1.3 and 2.5 million deaths of children aged 6 mo to 5 y could be prevented each year by proper vitamin A nutrition (Figure A1-2). Two million children have clinical or severe visual problems and more than 250000 children become blind as a result of VAD each year, one-half of whom die within a year of losing their sight. (FAO 2002). The chances of death from measles or diarrheal disease are greatly increased by VAD (FAO/WHO 2002). VAD also interacts with other nutrients; for example, iron metabolism is negatively affected and iron is not incorporated effectively into hemoglobin (Hodges and others 1978).

Many countries with a high prevalence of VAD rely on rice as a major source of energy. Rice does not contain β-carotene, the direct precursor of vitamin A. Attempts to provide biannual vitamin A supplements to all children less than 6 y of age have been difficult to sustain and are comparatively expensive (Bouis and others 2003). More recently, fortification of foods such as sugar and cereal flours with vitamin A has come to the fore. Lack of dietary diversity causes VAD, and historically, emphasis has been on increasing the intake of green leafy vegetables and yellow-orange fruits and vegetables to improve vitamin A intake. However, the β-carotene in green leafy vegetable is less well absorbed than that from yellow-orange fruits and roots or tubers, although the method of preparation, including the presence of dietary lipid, can improve bioefficacy (Yeum and Russell 2002; Haskell and others 2004). The Inst. of Medicine (2001) has proposed that 12 μg of β-carotene are required to provide 1 μg of vitamin A, while a conversion factor of 1 to 24 μg has been set for other provitamin A carotenoids, that is, β-cryptoxanthin and α-carotene.

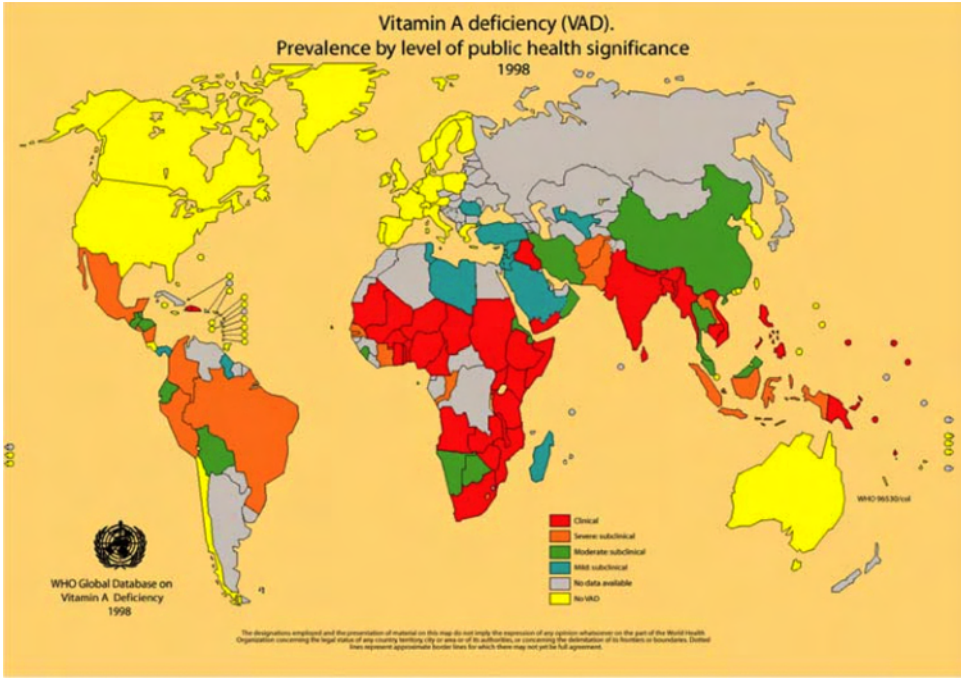

Figure A1-1—Vitamin A deficiency around the globe (FAO/WHO 2002).

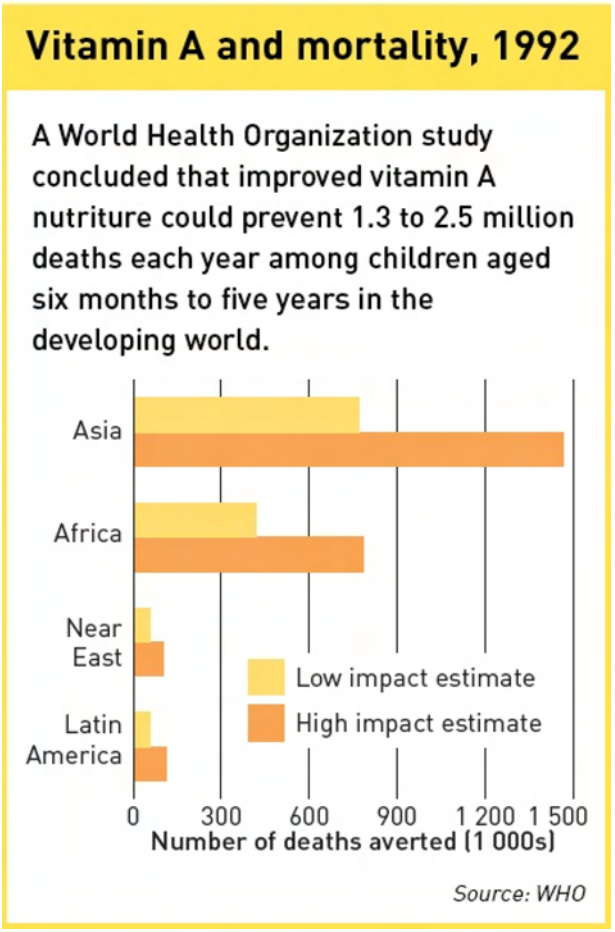

Figure A1-2—WHO estimates of childhood mortality (FAO 1992).

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Appendix 2: Biosynthesis of β-Carotene

The details of the biosynthesis of β-carotene and other carotenoids in plants have been reviewed elsewhere (Cunningham and Gantt 1998; Sandmann 2001; Taylor and Ramsay 2005). The initial steps in the biosynthesis of β-carotene involve intermediates that are also precursors to other products in plant cells. For example, the 5-carbon molecule (C5) isopentenyl pyrophosphate (IPP) is the building unit for the synthesis of various kinds of terpenoids, including β-carotenes, plant hormones, and other secondary metabolites and defense molecules. IPP is formed both in the cytosol, through the mevalonate pathway, and in plastids, through the deoxy-xylulose-phosphate pathway. In plastids, IPP serves as substrate for carotenoid biosynthesis, among others, and in the cytosol for some plant hormones and steroids.

Because IPP isomerase is present in plant plastids and its activity is the limiting step of carotenoid synthesis in etioplasts, it has been concluded that IPP is the major or only product of the 1-deoxyxylulose-5-phosphate pathway–-the reaction sequence leading to cyclic carotenoids (Albrecht and others 1994).

The 1st steps in the biosynthesis from IPP to β-carotene entail the formation of the C20 compound geranylgeranylphosphate (GGPP) by condensation of 4 C5-precursors in total. First, 1 IPP molecule is isomerized by the enzyme IPP isomerase into dimethylallyl-pyrophosphate (DMAPP). This activated form of IPP then reacts with 3 IPP molecules in 3 discrete reactions, leading to the formation of GGPP catalyzed by the enzyme GGPP synthase. Two molecules of GGPP are subsequently condensed to phytoene (C40) under the influence of the enzyme phytoene synthase.

Phytoene enters a series of dehydrogenation and ring formation reactions, resulting in the formation of β-carotene. Phytoene desaturase (PDS) and zeta carotene desaturase (ZDS)

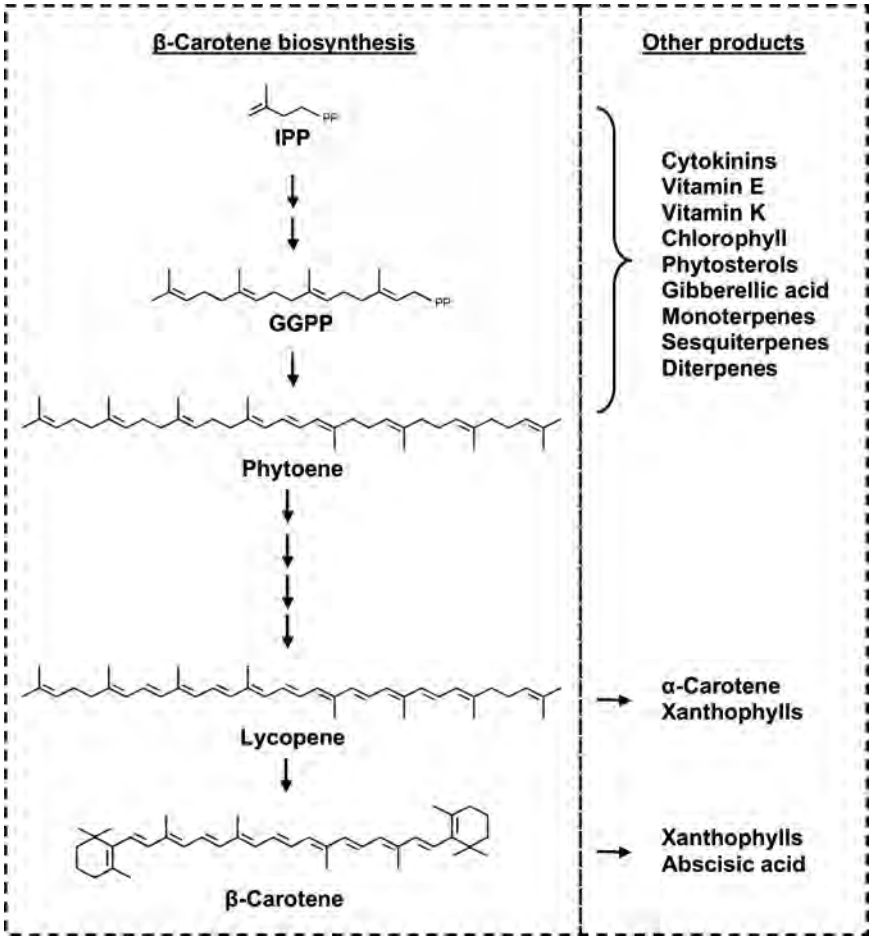

Figure A2-1—Biosynthesis of β-carotene in plants and other products originating from intermediates in the same pathway. [Based on "Biochemical Pathways" chart of Roche Applied Sciences (www. expasy.org/ tools/pathways/) and Taylor and Ramsay (2005)].

catalyze 4 dehydrogenation reactions, while carotenoid isomerase (CRTISO) catalyzes the *cis-trans* isomerization of cis double bonds, particularly the 15-*cis*-double bond between carbon atoms, so that ultimately all-*trans*-lycopene is formed. The enzyme lycopene β-cyclase (LCYB) converts lycopene into β-carotene through 2 cyclization reactions involving the formation of C6-rings at both ends of the molecule. In addition, β-carotene itself may be converted to xanthophylls, which are carotenoid molecules with hydroxylated rings. Further reactions of xanthophylls lead to, for example, formation of the plant growth regulator, abscisic acid (Cunningham and Gantt 1998; Taylor and Ramsay 2005).

Figure A2-1 provides a schematic overview of the pathway leading to biosynthesis of β-carotene and products derived from branches of the same pathway. Increasing the flux through this pathway toward β-carotene or intermediates may alter the biosynthesis of other metabolites. For example, Fray and others (1995) found that levels of intermediates, including phytoene, lycopene, and ζ-carotene, had increased in tomato plants expressing phytoene synthase. In addition, transgenic plants showed stunted growth due to decreased production of gibberellic acid-based plant growth regulators. Chlorophyll biosynthesis was decreased in these plants as well.

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Appendix 3: Physiology of β-Carotene

Intake of β-Carotene

Beta-carotene is an important provitamin A carotenoid, that is, precursor of vitamin A, but is not an essential nutrient and has no dietary reference value. Dietary intakes are expressed as total micrograms of retinol equivalents (RE), in which 12 μg β-carotene is equivalent nutritionally to 1 μg RE. The FAO/WHO (2002) recommended safe dietary intake of vitamin A is 375 μg/d for infants, 400 to 450 μg/d for children 1 to 6 y, 800 μg/d for pregnant women, and 850 μg/d for lactating women.

Provitamin A carotenoids are generally ingested from plant foods and can be biologically transformed to vitamin A. Globally, about 60% of dietary vitamin A comes from provitamins A. Many factors influence the absorption and utilization of provitamin A, such as the vitamin A status of the individual; the amount, type, and physical form of the carotenoids in the diet; the intake of fat, vitamin A, and fiber; the individual's protein and zinc status; and the presence of certain diseases and parasitic infections (de Pee and West 1996). Between 10% and 90% of all dietary β-carotene is absorbed, and absorption decreases as intake increases. Bioavailability is reduced in very low-fat diets.

Metabolism of β-Carotene

The metabolism of β-carotene in humans and animals is well documented, with excellent reviews by Olson (1996), Parker (1997), Burri and Clifford (2004), and Bendich (2004).

In humans, dietary fat and bile salts facilitate the absorption of β-carotene that occurs in the upper small intestine. Absorption occurs via incorporation into multilamellar lipid micelles, although a proportion of absorbed β-carotene is converted to retinol within intestinal mucosal cells. Unaltered β-carotene is transported via the lymph to the plasma where it is associated with lipoproteins. Tissue uptake and distribution are not well characterized. When the intake of β-carotene is consistently high, long-term accumulation occurs preferentially in adipose tissues.

Experiments in rats have shown that the conversion of β-carotene to retinol is regulated by the levels of β-carotene and of preformed vitamin A. *In vitro* studies have shown that other β-carotene derivatives may also occur, but their biological activity, and whether they are synthesized in vivo, is unknown. Carotenoid absorption and metabolism vary considerably between animal species (Lee and others 1999). No single species is a good model for studying biokinetics and metabolism of β-carotene in humans. The rat is unsuitable because it is highly efficient at converting β-carotene to vitamin A, and significant levels of unaltered β-carotene are absorbed only when very high doses are given for a prolonged time. The preruminant calf, the ferret, and the Mongolian gerbil are more useful models, although there are many differences in carotenoid absorption, distribution, and metabolism between these animals and humans.

Toxicity of Carotene

Dietary β-carotene has not been shown to be toxic in humans (Expert Group on Vitamins and Minerals 2003). Hypercarotenaemia (high plasma β-carotene) has not been associated with adverse effects other than reversible yellowing of the skin, known as hypercarotenodermia. Long-term oral β-carotene therapy in doses up to 300 μg/d showed no toxic effects in individuals with erythropoietic protoporphyria. Vitamin A toxicity does not occur because the metabolic conversion is regulated by vitamin A status. Reproductive toxicity or teratogenicity associated with high β-carotene intake, either before or during pregnancy, has not been reported. Two large-scale supplementation trials testing the hypothesis that β-carotene supplementation in smokers would reduce the incidence of cancer have shown an association of high dose β-carotene supplementation (20 to 30 mg/d) with increased incidence of lung cancer in smokers and asbestos-exposed individuals (Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn and others 1996). No statistically significant differences in other cancer types were observed in these studies.

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Appendix 4: Protein-Energy Malnutrition

Malnutrition includes both under- and overnutrition. Undernutrition is the general term for an inadequate intake of energy or specific vitamins and minerals that are collectively known as micronutrients. Overnutrition is the opposite. This section focuses on undernutrition, specifically protein-energy malnutrition (PEM). PEM syndrome results from inadequate food intake and is characterized by energy deficits, due to a reduced intake of all macronutrients and often many micronutrients as well. Clinically, PEM manifests as emaciation (marasmus), edema (kwashiorkor), or a combination of the two (marasmic–kwashiorkor). The form depends on the balance of nonprotein and protein sources of energy. Marasmus occurs when energy intake is insufficient to meet requirements and the body is forced to draw on its own stores: first glycogen, then skeletal muscle and triglycerides in fat depots. Consequently, the individual becomes thin from loss of muscle and body fat. In kwashiorkor, dietary protein deficiency is usually more marked than the energy deficiency, and this results in decreased protein synthesis and edema. Children with marasmic–kwashiorkor have some edema and more body fat than those with marasmus.

Marasmus is the predominant form of PEM in most developing countries and can affect all age groups. Kwashiorkor is less common and is usually manifested as marasmic–kwashiorkor, especially in young children. It tends to occur where the staple foods are low in protein and excessively starchy (for example, yam [Dioscorea], cassava, sweetpotato, taro, plantain, and banana).

The assessment of PEM uses indices derived from measurements of body stature or length, weight, age, and presence or absence of edema that are compared with a reference population considered to have normal growth characteristics---usually the United States, although this has been questioned. The indices are underweight (low weight for age), stunting (low height for age), and wasting (low weight for height). National and regional level data on nutritional status have been compiled systematically for more than 20 y by the WHO Global Database on Child Growth and Malnutrition, which represents most of the children worldwide (de Onis and others 2004). Because the population in Asia is larger than elsewhere in the world, the number of undernourished is greatest in Asia, followed by Africa, although the proportion that are undernourished is greatest in Africa (de Onis and others 2004).

Views on the causes of PEM have evolved over time. By the 1960s, for example, it was known that the supply of protein in cereal-based diets was adequate (Hegsted and others 1946). The FAO nevertheless considered protein deficiency to be serious and widespread in developing countries. This was based on the observations that kwashiorkor, which was common among 1- to 4-y-old children in developing countries, (1) responded to therapy that included relatively high-protein supplements and (2) caused liver damage. Because liver cirrhosis was common among adults in Africa, the FAO thought this too indicated that African diets remained protein-deficient throughout life and the same might also be true in all developing countries. Milk and milk powder were expensive and in short supply. Consequently, much work was carried out to develop and test economical alternatives to milk powder based on locally available cereals and oilseed flours, fish protein concentrate, and single-cell protein from fermenting microorganisms. The work ended once it was realized that most children with kwashiorkor had been raised on diets that were as deficient in energy as they were in protein. The diets were also too bulky, which meant children could only eat a limited and inadequate amount (Whitehead 1973). Consensus was reached that the solution was to provide more high-energy foods and correct electrolyte deficiencies rather than concentrate just on protein (Waterlow 1961; McLaren 1974; Carpenter 1994), although it was recognized that diets based on staples such as cassava, bananas, and yams (Dioscorea) that are both very bulky and in low in protein present special challenges (Nicol 1971). In addition, initial treatment for severe PEM requires comparatively low levels of protein with a high biological value to ensure optimal utilization of protein for tissue maintenance and to prevent overloading the impaired metabolic system with superfluous amino acids and their nitrogenous metabolites. Following this initial treatment, high-protein diets such as milk-based formula can be used to stimulate growth and recovery (for example, Müller and Krawinkel 2005).

The importance of good nutrition starts at conception because fetal growth and development lay the foundation for health at all later ages. A fetus depends on the mother's ability to deliver nutrients, and this is greatly influenced by her nutritional state at the time she becomes pregnant as well as throughout pregnancy. Poor fetal growth and development have major consequences for the offspring, and these also have implications for health in the neonatal period, infancy, childhood, adolescence, and later life. Undernutrition in early childhood has serious consequences as these children are not only more prone to illness, but they also tend to have more severe illnesses than well-nourished children. Undernourished children are also at greater risk of dying. Undernutrition in school age children, much of which probably started in early childhood, adversely affects school attendance, performance, and learning. A stunted girl is more likely to become a stunted adolescent and later a stunted woman. Apart from direct effects on her health and productivity, undernutrition in an adult woman increases the chance that her children will be born with low birth weight, and the cycle begins again (United Nations Standing Committee on Nutrition 2004). Interventions to control undernutrition are therefore needed at different stages in the life cycle. These include improved child care and feeding practices for infants and young children; the provision of specific micronutrients through food fortification, biofortification, and pharmaceutical supplementation; improved access to health care; household

food security; good hygiene and sanitation; and universal female education.

The WHO global strategy for feeding infants and young children highlights the importance of good feeding practices during the transition period between exclusive breastfeeding and full weaning, when breastfed children also need complementary food. In addition to being safe and timely, the diet must be adequate—defined as providing sufficient energy, protein, and micronutrients to meet a growing child's nutritional needs (WHO 2002). Children need a variety of foods to ensure that nutrient needs are met. Diets that lack animal source foods (meat, poultry, fish or eggs, plus milk products) cannot meet the nutrient requirements for children ages 6 to 24 mo unless fortified foods or supplements are used. If milk and other animal source foods are not taken in adequate amounts, both grains and legumes should be consumed daily, preferably within the same meal, to ensure adequate protein quality (WHO 2004). In addition to the availability of these foods, their accessibility to the population depending on, for example, infrastructure and economic resources needs to be considered.

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Appendix 5: Deregulation (Excerpted from Al-Babili and Beyer 2005)

Deregulation is one term used for the process that eventually leads to the registration of a product and the granting of its unlimited commercial or noncommercial use. The national authorities of the respective countries in which the product is intended for use govern deregulation of GMO plants and the foods derived from these. The process is driven by a science-based risk assessment of the intended product and the environments into which it will be released. Current data requirements can be extensive and are comprehensive, and require the submission of publication quality data reports. The development of these materials can be time- consuming and costly. The studies required to compile a regulatory dossier fall into 2 categories: event-dependent and event-independent studies.

Event-independent studies can include biochemical analysis (function, specificity and mode of action), studies on toxicity and the allergenic potential of the newly expressed proteins. In addition, dietary exposure modeling and bioavailability studies can be conducted with the newly expressed proteins and, in the case of enzymes, with the products produced by catalysis.

Event-dependent studies vary by country and can encompass the characterization of the quality of integration, such as copy number, the determination of the site of integration in the host genome, demonstration of the absence of gene disruption, completeness of integrated DNA sequences, and the presence of the marker gene at the same locus and absence of vector DNA sequences. Phenotypic and biochemical evidence for trait stability, including the monitoring of gene expression levels at key growth stages and Mendelian inheritance over several generations, are usually components of these studies. Field performance of typical agronomic traits, such as yield, and pest and disease resistance, is an additional issue. The assessment is also based on the comparison of the compositional analyses from materials harvested at different locations with the equivalent nontransgenic crop (and food) comparator. Event-dependent regulatory data are usually not gathered until the developer is satisfied with the technical performance of the product and a few events (usually one) have been chosen. Assessment of the potential risks of GM crops is on a case-by-case basis within a scientific framework. A GMO crop producing β-carotene must be viewed differently than a crop that produces an insecticide. Similarly, GR events expressing a phytoene synthase from maize, a widely consumed crop, might be viewed differently than one using the enzyme from a nonfood plant, such as daffodil.

From a scientific point of view it is often hard to understand why randomly mutagenized crop plants can enter into breeding lines easily, whereas GMOs, with considerably fewer genetic modifications, have a much more rigorous assessment. To have rational, science-based regulatory requirements it would be necessary to realize the benefits (not only potential risks) that GMO crops can provide (see lecture by Ingo Potrykus at http://www.syngentafoundation.com/golden_rice/index.htm). It is unhelpful to raise the regulatory requirements to the point where anything that appears technically feasible is being requested or is being offered to be applied (Kuiper and others

2001), with the only justification that the genetically modified organism involved is little understood. This strategy can raise costs to unaffordable levels. We do not understand bred varieties with their complex genomic changes much better at the molecular level, but we tend to consider the traditional way of producing new varieties safe in contrast to the novel way of producing GMOs even though this is not based on any rational evaluation. It is the final product and not the technology used to produce it that should be scrutinized.

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Appendix 1: Vitamin A Deficiency: A Global Risk

Vitamin A deficiency (VAD) is a global public health challenge, particularly in infants, children, and pregnant and lactating women because of the extra requirements to support fetal, infant, and child growth (Figure A1-1). Deficiency can result in reduced resistance to infection, impaired cellular differentiation, xerophthalmia, and ultimately blindness and death; it is also associated with anemia. More than 100 million children and as many as 7 million pregnant women living in over 100 countries have VAD (West 2002; WHO 2003). It is estimated that between 1.3 and 2.5 million deaths of children aged 6 mo to 5 y could be prevented each year by proper vitamin A nutrition (Figure A1-2). Two million children have clinical or severe visual problems and more than 250000 children become blind as a result of VAD each year, one-half of whom die within a year of losing their sight. (FAO 2002). The chances of death from measles or diarrheal disease are greatly increased by VAD (FAO/WHO 2002). VAD also interacts with other nutrients; for example, iron metabolism is negatively affected and iron is not incorporated effectively into hemoglobin (Hodges and others 1978).

Many countries with a high prevalence of VAD rely on rice as a major source of energy. Rice does not contain β-carotene, the direct precursor of vitamin A. Attempts to provide biannual vitamin A supplements to all children less than 6 y of age have been difficult to sustain and are comparatively expensive (Bouis and others 2003). More recently, fortification of foods such as sugar and cereal flours with vitamin A has come to the fore. Lack of dietary diversity causes VAD, and historically, emphasis has been on increasing the intake of green leafy vegetables and yellow-orange fruits and vegetables to improve vitamin A intake. However, the β-carotene in green leafy vegetable is less well absorbed than that from yellow-orange fruits and roots or tubers, although the method of preparation, including the presence of dietary lipid, can improve bioefficacy (Yeum and Russell 2002; Haskell and others 2004). The Inst. of Medicine (2001) has proposed that 12 μg of β-carotene are required to provide 1 μg of vitamin A, while a conversion factor of 1 to 24 μg has been set for other provitamin A carotenoids, that is, β-cryptoxanthin and α-carotene.

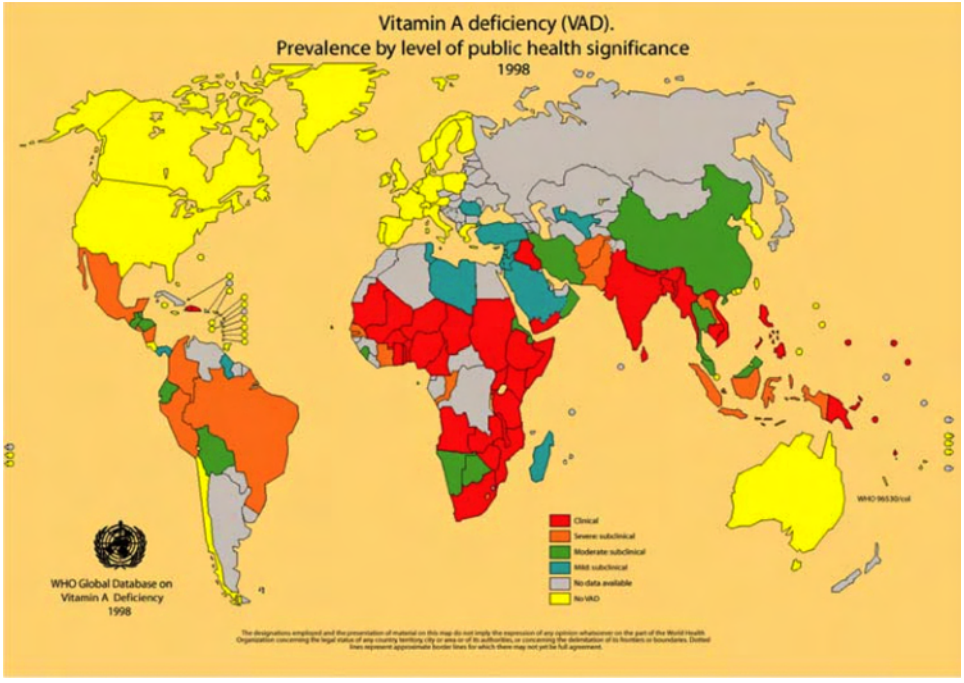

Figure A1-1—Vitamin A deficiency around the globe (FAO/WHO 2002).

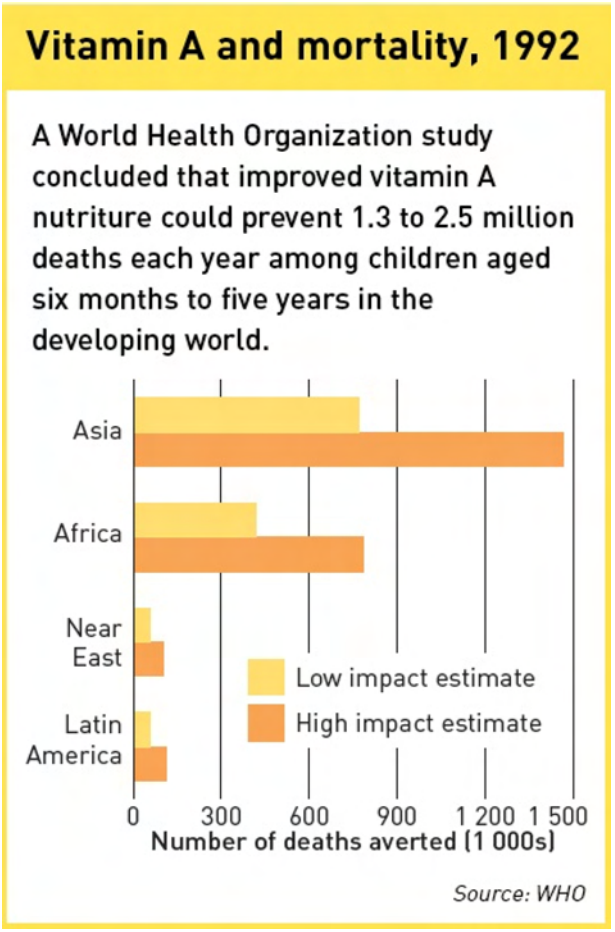

Figure A1-2—WHO estimates of childhood mortality (FAO 1992).

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Appendix 2: Biosynthesis of β-Carotene

The details of the biosynthesis of β-carotene and other carotenoids in plants have been reviewed elsewhere (Cunningham and Gantt 1998; Sandmann 2001; Taylor and Ramsay 2005). The initial steps in the biosynthesis of β-carotene involve intermediates that are also precursors to other products in plant cells. For example, the 5-carbon molecule (C5) isopentenyl pyrophosphate (IPP) is the building unit for the synthesis of various kinds of terpenoids, including β-carotenes, plant hormones, and other secondary metabolites and defense molecules. IPP is formed both in the cytosol, through the mevalonate pathway, and in plastids, through the deoxy-xylulose-phosphate pathway. In plastids, IPP serves as substrate for carotenoid biosynthesis, among others, and in the cytosol for some plant hormones and steroids.

Because IPP isomerase is present in plant plastids and its activity is the limiting step of carotenoid synthesis in etioplasts, it has been concluded that IPP is the major or only product of the 1-deoxyxylulose-5-phosphate pathway–-the reaction sequence leading to cyclic carotenoids (Albrecht and others 1994).

The 1st steps in the biosynthesis from IPP to β-carotene entail the formation of the C20 compound geranylgeranylphosphate (GGPP) by condensation of 4 C5-precursors in total. First, 1 IPP molecule is isomerized by the enzyme IPP isomerase into dimethylallyl-pyrophosphate (DMAPP). This activated form of IPP then reacts with 3 IPP molecules in 3 discrete reactions, leading to the formation of GGPP catalyzed by the enzyme GGPP synthase. Two molecules of GGPP are subsequently condensed to phytoene (C40) under the influence of the enzyme phytoene synthase.

Phytoene enters a series of dehydrogenation and ring formation reactions, resulting in the formation of β-carotene. Phytoene desaturase (PDS) and zeta carotene desaturase (ZDS)

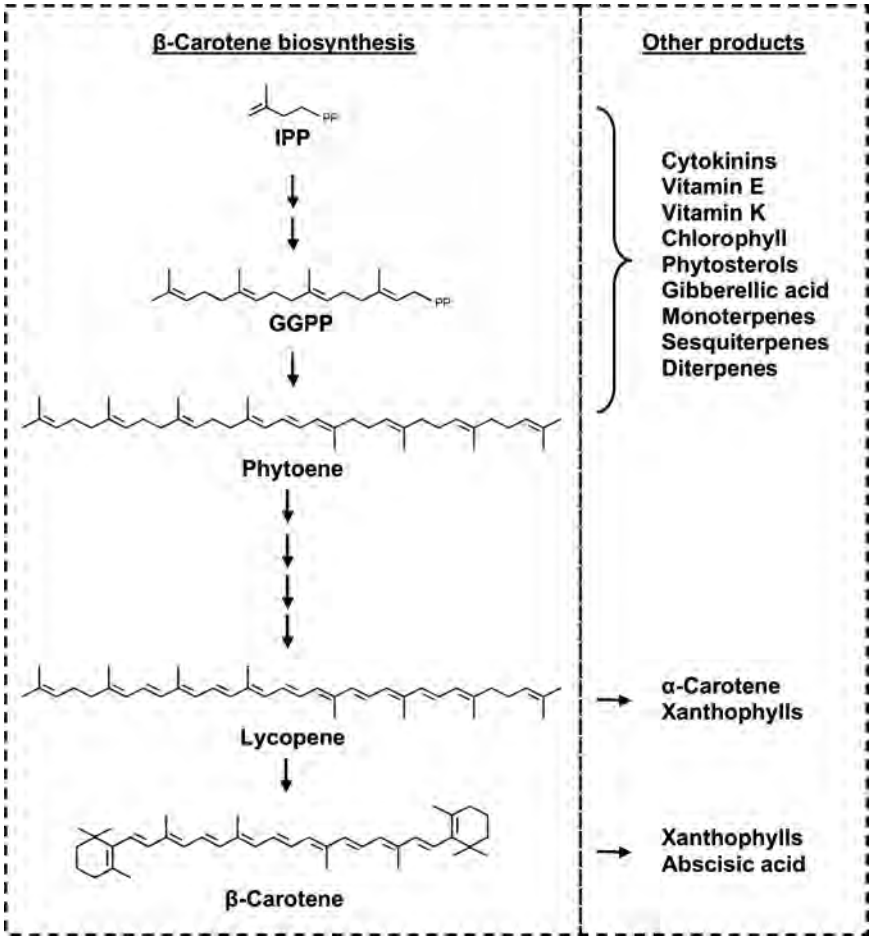

Figure A2-1—Biosynthesis of β-carotene in plants and other products originating from intermediates in the same pathway. [Based on "Biochemical Pathways" chart of Roche Applied Sciences (www. expasy.org/ tools/pathways/) and Taylor and Ramsay (2005)].

catalyze 4 dehydrogenation reactions, while carotenoid isomerase (CRTISO) catalyzes the *cis-trans* isomerization of cis double bonds, particularly the 15-*cis*-double bond between carbon atoms, so that ultimately all-*trans*-lycopene is formed. The enzyme lycopene β-cyclase (LCYB) converts lycopene into β-carotene through 2 cyclization reactions involving the formation of C6-rings at both ends of the molecule. In addition, β-carotene itself may be converted to xanthophylls, which are carotenoid molecules with hydroxylated rings. Further reactions of xanthophylls lead to, for example, formation of the plant growth regulator, abscisic acid (Cunningham and Gantt 1998; Taylor and Ramsay 2005).

Figure A2-1 provides a schematic overview of the pathway leading to biosynthesis of β-carotene and products derived from branches of the same pathway. Increasing the flux through this pathway toward β-carotene or intermediates may alter the biosynthesis of other metabolites. For example, Fray and others (1995) found that levels of intermediates, including phytoene, lycopene, and ζ-carotene, had increased in tomato plants expressing phytoene synthase. In addition, transgenic plants showed stunted growth due to decreased production of gibberellic acid-based plant growth regulators. Chlorophyll biosynthesis was decreased in these plants as well.

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Provitamin A carotenoids are generally ingested from plant foods and can be biologically transformed to vitamin A. Globally, about 60% of dietary vitamin A comes from provitamins A. Many factors influence the absorption and utilization of provitamin A, such as the vitamin A status of the individual; the amount, type, and physical form of the carotenoids in the diet; the intake of fat, vitamin A, and fiber; the individual's protein and zinc status; and the presence of certain diseases and parasitic infections (de Pee and West 1996). Between 10% and 90% of all dietary β-carotene is absorbed, and absorption decreases as intake increases. Bioavailability is reduced in very low-fat diets.

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Appendix 4: Protein-Energy Malnutrition

Malnutrition includes both under- and overnutrition. Undernutrition is the general term for an inadequate intake of energy or specific vitamins and minerals that are collectively known as micronutrients. Overnutrition is the opposite. This section focuses on undernutrition, specifically protein-energy malnutrition (PEM). PEM syndrome results from inadequate food intake and is characterized by energy deficits, due to a reduced intake of all macronutrients and often many micronutrients as well. Clinically, PEM manifests as emaciation (marasmus), edema (kwashiorkor), or a combination of the two (marasmic–kwashiorkor). The form depends on the balance of nonprotein and protein sources of energy. Marasmus occurs when energy intake is insufficient to meet requirements and the body is forced to draw on its own stores: first glycogen, then skeletal muscle and triglycerides in fat depots. Consequently, the individual becomes thin from loss of muscle and body fat. In kwashiorkor, dietary protein deficiency is usually more marked than the energy deficiency, and this results in decreased protein synthesis and edema. Children with marasmic–kwashiorkor have some edema and more body fat than those with marasmus.

Marasmus is the predominant form of PEM in most developing countries and can affect all age groups. Kwashiorkor is less common and is usually manifested as marasmic–kwashiorkor, especially in young children. It tends to occur where the staple foods are low in protein and excessively starchy (for example, yam [Dioscorea], cassava, sweetpotato, taro, plantain, and banana).

The assessment of PEM uses indices derived from measurements of body stature or length, weight, age, and presence or absence of edema that are compared with a reference population considered to have normal growth characteristics---usually the United States, although this has been questioned. The indices are underweight (low weight for age), stunting (low height for age), and wasting (low weight for height). National and regional level data on nutritional status have been compiled systematically for more than 20 y by the WHO Global Database on Child Growth and Malnutrition, which represents most of the children worldwide (de Onis and others 2004). Because the population in Asia is larger than elsewhere in the world, the number of undernourished is greatest in Asia, followed by Africa, although the proportion that are undernourished is greatest in Africa (de Onis and others 2004).

Views on the causes of PEM have evolved over time. By the 1960s, for example, it was known that the supply of protein in cereal-based diets was adequate (Hegsted and others 1946). The FAO nevertheless considered protein deficiency to be serious and widespread in developing countries. This was based on the observations that kwashiorkor, which was common among 1- to 4-y-old children in developing countries, (1) responded to therapy that included relatively high-protein supplements and (2) caused liver damage. Because liver cirrhosis was common among adults in Africa, the FAO thought this too indicated that African diets remained protein-deficient throughout life and the same might also be true in all developing countries. Milk and milk powder were expensive and in short supply. Consequently, much work was carried out to develop and test economical alternatives to milk powder based on locally available cereals and oilseed flours, fish protein concentrate, and single-cell protein from fermenting microorganisms. The work ended once it was realized that most children with kwashiorkor had been raised on diets that were as deficient in energy as they were in protein. The diets were also too bulky, which meant children could only eat a limited and inadequate amount (Whitehead 1973). Consensus was reached that the solution was to provide more high-energy foods and correct electrolyte deficiencies rather than concentrate just on protein (Waterlow 1961; McLaren 1974; Carpenter 1994), although it was recognized that diets based on staples such as cassava, bananas, and yams (Dioscorea) that are both very bulky and in low in protein present special challenges (Nicol 1971). In addition, initial treatment for severe PEM requires comparatively low levels of protein with a high biological value to ensure optimal utilization of protein for tissue maintenance and to prevent overloading the impaired metabolic system with superfluous amino acids and their nitrogenous metabolites. Following this initial treatment, high-protein diets such as milk-based formula can be used to stimulate growth and recovery (for example, Müller and Krawinkel 2005).

The importance of good nutrition starts at conception because fetal growth and development lay the foundation for health at all later ages. A fetus depends on the mother's ability to deliver nutrients, and this is greatly influenced by her nutritional state at the time she becomes pregnant as well as throughout pregnancy. Poor fetal growth and development have major consequences for the offspring, and these also have implications for health in the neonatal period, infancy, childhood, adolescence, and later life. Undernutrition in early childhood has serious consequences as these children are not only more prone to illness, but they also tend to have more severe illnesses than well-nourished children. Undernourished children are also at greater risk of dying. Undernutrition in school age children, much of which probably started in early childhood, adversely affects school attendance, performance, and learning. A stunted girl is more likely to become a stunted adolescent and later a stunted woman. Apart from direct effects on her health and productivity, undernutrition in an adult woman increases the chance that her children will be born with low birth weight, and the cycle begins again (United Nations Standing Committee on Nutrition 2004). Interventions to control undernutrition are therefore needed at different stages in the life cycle. These include improved child care and feeding practices for infants and young children; the provision of specific micronutrients through food fortification, biofortification, and pharmaceutical supplementation; improved access to health care; household

food security; good hygiene and sanitation; and universal female education.

The WHO global strategy for feeding infants and young children highlights the importance of good feeding practices during the transition period between exclusive breastfeeding and full weaning, when breastfed children also need complementary food. In addition to being safe and timely, the diet must be adequate—defined as providing sufficient energy, protein, and micronutrients to meet a growing child's nutritional needs (WHO 2002). Children need a variety of foods to ensure that nutrient needs are met. Diets that lack animal source foods (meat, poultry, fish or eggs, plus milk products) cannot meet the nutrient requirements for children ages 6 to 24 mo unless fortified foods or supplements are used. If milk and other animal source foods are not taken in adequate amounts, both grains and legumes should be consumed daily, preferably within the same meal, to ensure adequate protein quality (WHO 2004). In addition to the availability of these foods, their accessibility to the population depending on, for example, infrastructure and economic resources needs to be considered.

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Appendix 5: Deregulation (Excerpted from Al-Babili and Beyer 2005)

Deregulation is one term used for the process that eventually leads to the registration of a product and the granting of its unlimited commercial or noncommercial use. The national authorities of the respective countries in which the product is intended for use govern deregulation of GMO plants and the foods derived from these. The process is driven by a science-based risk assessment of the intended product and the environments into which it will be released. Current data requirements can be extensive and are comprehensive, and require the submission of publication quality data reports. The development of these materials can be time- consuming and costly. The studies required to compile a regulatory dossier fall into 2 categories: event-dependent and event-independent studies.

Event-independent studies can include biochemical analysis (function, specificity and mode of action), studies on toxicity and the allergenic potential of the newly expressed proteins. In addition, dietary exposure modeling and bioavailability studies can be conducted with the newly expressed proteins and, in the case of enzymes, with the products produced by catalysis.

Event-dependent studies vary by country and can encompass the characterization of the quality of integration, such as copy number, the determination of the site of integration in the host genome, demonstration of the absence of gene disruption, completeness of integrated DNA sequences, and the presence of the marker gene at the same locus and absence of vector DNA sequences. Phenotypic and biochemical evidence for trait stability, including the monitoring of gene expression levels at key growth stages and Mendelian inheritance over several generations, are usually components of these studies. Field performance of typical agronomic traits, such as yield, and pest and disease resistance, is an additional issue. The assessment is also based on the comparison of the compositional analyses from materials harvested at different locations with the equivalent nontransgenic crop (and food) comparator. Event-dependent regulatory data are usually not gathered until the developer is satisfied with the technical performance of the product and a few events (usually one) have been chosen. Assessment of the potential risks of GM crops is on a case-by-case basis within a scientific framework. A GMO crop producing β-carotene must be viewed differently than a crop that produces an insecticide. Similarly, GR events expressing a phytoene synthase from maize, a widely consumed crop, might be viewed differently than one using the enzyme from a nonfood plant, such as daffodil.

From a scientific point of view it is often hard to understand why randomly mutagenized crop plants can enter into breeding lines easily, whereas GMOs, with considerably fewer genetic modifications, have a much more rigorous assessment. To have rational, science-based regulatory requirements it would be necessary to realize the benefits (not only potential risks) that GMO crops can provide (see lecture by Ingo Potrykus at http://www.syngentafoundation.com/golden_rice/index.htm). It is unhelpful to raise the regulatory requirements to the point where anything that appears technically feasible is being requested or is being offered to be applied (Kuiper and others

2001), with the only justification that the genetically modified organism involved is little understood. This strategy can raise costs to unaffordable levels. We do not understand bred varieties with their complex genomic changes much better at the molecular level, but we tend to consider the traditional way of producing new varieties safe in contrast to the novel way of producing GMOs even though this is not based on any rational evaluation. It is the final product and not the technology used to produce it that should be scrutinized.

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Appendix 1: Vitamin A Deficiency: A Global Risk

Vitamin A deficiency (VAD) is a global public health challenge, particularly in infants, children, and pregnant and lactating women because of the extra requirements to support fetal, infant, and child growth (Figure A1-1). Deficiency can result in reduced resistance to infection, impaired cellular differentiation, xerophthalmia, and ultimately blindness and death; it is also associated with anemia. More than 100 million children and as many as 7 million pregnant women living in over 100 countries have VAD (West 2002; WHO 2003). It is estimated that between 1.3 and 2.5 million deaths of children aged 6 mo to 5 y could be prevented each year by proper vitamin A nutrition (Figure A1-2). Two million children have clinical or severe visual problems and more than 250000 children become blind as a result of VAD each year, one-half of whom die within a year of losing their sight. (FAO 2002). The chances of death from measles or diarrheal disease are greatly increased by VAD (FAO/WHO 2002). VAD also interacts with other nutrients; for example, iron metabolism is negatively affected and iron is not incorporated effectively into hemoglobin (Hodges and others 1978).

Many countries with a high prevalence of VAD rely on rice as a major source of energy. Rice does not contain β-carotene, the direct precursor of vitamin A. Attempts to provide biannual vitamin A supplements to all children less than 6 y of age have been difficult to sustain and are comparatively expensive (Bouis and others 2003). More recently, fortification of foods such as sugar and cereal flours with vitamin A has come to the fore. Lack of dietary diversity causes VAD, and historically, emphasis has been on increasing the intake of green leafy vegetables and yellow-orange fruits and vegetables to improve vitamin A intake. However, the β-carotene in green leafy vegetable is less well absorbed than that from yellow-orange fruits and roots or tubers, although the method of preparation, including the presence of dietary lipid, can improve bioefficacy (Yeum and Russell 2002; Haskell and others 2004). The Inst. of Medicine (2001) has proposed that 12 μg of β-carotene are required to provide 1 μg of vitamin A, while a conversion factor of 1 to 24 μg has been set for other provitamin A carotenoids, that is, β-cryptoxanthin and α-carotene.

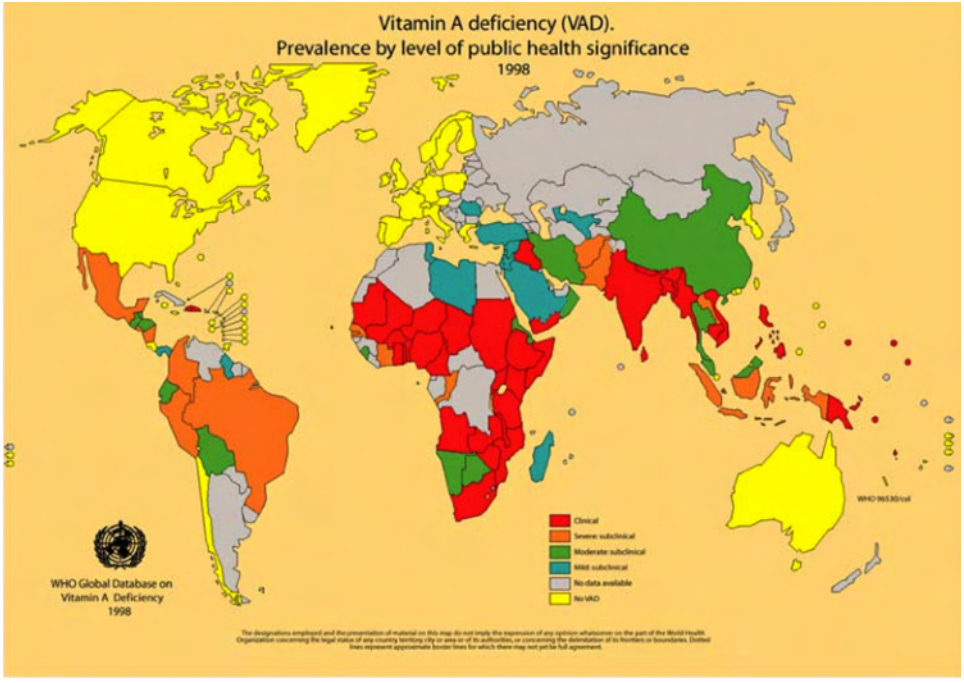

Figure A1-1—Vitamin A deficiency around the globe (FAO/WHO 2002).

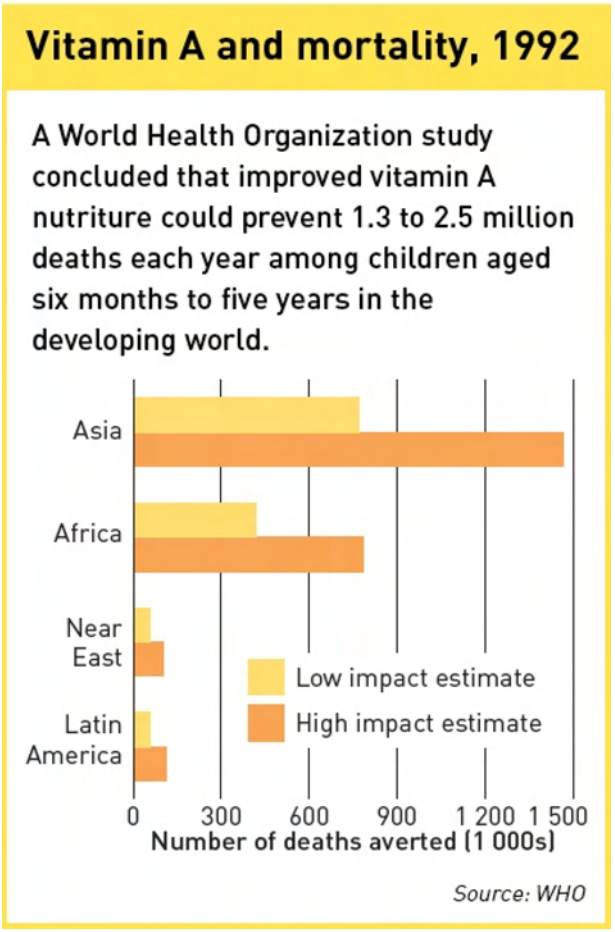

Figure A1-2—WHO estimates of childhood mortality (FAO 1992).

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Appendix 2: Biosynthesis of β-Carotene

The details of the biosynthesis of β-carotene and other carotenoids in plants have been reviewed elsewhere (Cunningham and Gantt 1998; Sandmann 2001; Taylor and Ramsay 2005). The initial steps in the biosynthesis of β-carotene involve intermediates that are also precursors to other products in plant cells. For example, the 5-carbon molecule (C5) isopentenyl pyrophosphate (IPP) is the building unit for the synthesis of various kinds of terpenoids, including β-carotenes, plant hormones, and other secondary metabolites and defense molecules. IPP is formed both in the cytosol, through the mevalonate pathway, and in plastids, through the deoxy-xylulose-phosphate pathway. In plastids, IPP serves as substrate for carotenoid biosynthesis, among others, and in the cytosol for some plant hormones and steroids.

Because IPP isomerase is present in plant plastids and its activity is the limiting step of carotenoid synthesis in etioplasts, it has been concluded that IPP is the major or only product of the 1-deoxyxylulose-5-phosphate pathway–-the reaction sequence leading to cyclic carotenoids (Albrecht and others 1994).

The 1st steps in the biosynthesis from IPP to β-carotene entail the formation of the C20 compound geranylgeranylphosphate (GGPP) by condensation of 4 C5-precursors in total. First, 1 IPP molecule is isomerized by the enzyme IPP isomerase into dimethylallyl-pyrophosphate (DMAPP). This activated form of IPP then reacts with 3 IPP molecules in 3 discrete reactions, leading to the formation of GGPP catalyzed by the enzyme GGPP synthase. Two molecules of GGPP are subsequently condensed to phytoene (C40) under the influence of the enzyme phytoene synthase.

Phytoene enters a series of dehydrogenation and ring formation reactions, resulting in the formation of β-carotene. Phytoene desaturase (PDS) and zeta carotene desaturase (ZDS)

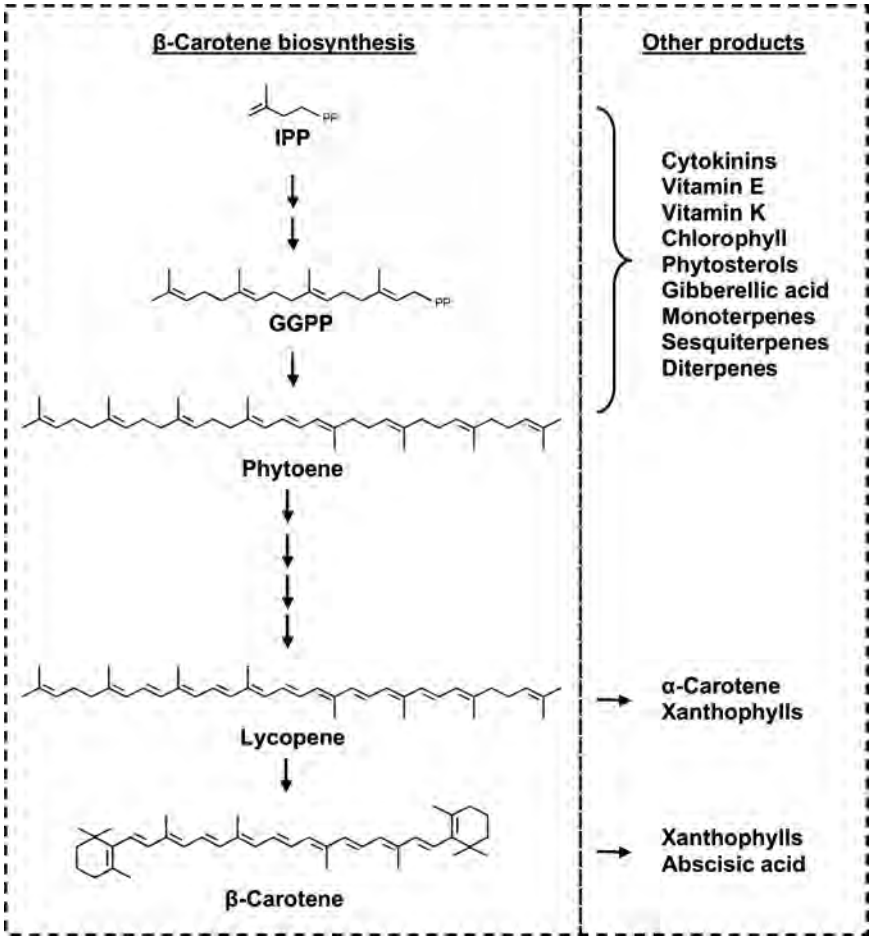

Figure A2-1—Biosynthesis of β-carotene in plants and other products originating from intermediates in the same pathway. [Based on "Biochemical Pathways" chart of Roche Applied Sciences (www. expasy.org/ tools/pathways/) and Taylor and Ramsay (2005)].

catalyze 4 dehydrogenation reactions, while carotenoid isomerase (CRTISO) catalyzes the *cis-trans* isomerization of cis double bonds, particularly the 15-*cis*-double bond between carbon atoms, so that ultimately all-*trans*-lycopene is formed. The enzyme lycopene β-cyclase (LCYB) converts lycopene into β-carotene through 2 cyclization reactions involving the formation of C6-rings at both ends of the molecule. In addition, β-carotene itself may be converted to xanthophylls, which are carotenoid molecules with hydroxylated rings. Further reactions of xanthophylls lead to, for example, formation of the plant growth regulator, abscisic acid (Cunningham and Gantt 1998; Taylor and Ramsay 2005).

Figure A2-1 provides a schematic overview of the pathway leading to biosynthesis of β-carotene and products derived from branches of the same pathway. Increasing the flux through this pathway toward β-carotene or intermediates may alter the biosynthesis of other metabolites. For example, Fray and others (1995) found that levels of intermediates, including phytoene, lycopene, and ζ-carotene, had increased in tomato plants expressing phytoene synthase. In addition, transgenic plants showed stunted growth due to decreased production of gibberellic acid-based plant growth regulators. Chlorophyll biosynthesis was decreased in these plants as well.

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Appendix 3: Physiology of β-Carotene

Intake of β-Carotene

Beta-carotene is an important provitamin A carotenoid, that is, precursor of vitamin A, but is not an essential nutrient and has no dietary reference value. Dietary intakes are expressed as total micrograms of retinol equivalents (RE), in which 12 μg β-carotene is equivalent nutritionally to 1 μg RE. The FAO/WHO (2002) recommended safe dietary intake of vitamin A is 375 μg/d for infants, 400 to 450 μg/d for children 1 to 6 y, 800 μg/d for pregnant women, and 850 μg/d for lactating women.

Provitamin A carotenoids are generally ingested from plant foods and can be biologically transformed to vitamin A. Globally, about 60% of dietary vitamin A comes from provitamins A. Many factors influence the absorption and utilization of provitamin A, such as the vitamin A status of the individual; the amount, type, and physical form of the carotenoids in the diet; the intake of fat, vitamin A, and fiber; the individual's protein and zinc status; and the presence of certain diseases and parasitic infections (de Pee and West 1996). Between 10% and 90% of all dietary β-carotene is absorbed, and absorption decreases as intake increases. Bioavailability is reduced in very low-fat diets.

Metabolism of β-Carotene

The metabolism of β-carotene in humans and animals is well documented, with excellent reviews by Olson (1996), Parker (1997), Burri and Clifford (2004), and Bendich (2004).

In humans, dietary fat and bile salts facilitate the absorption of β-carotene that occurs in the upper small intestine. Absorption occurs via incorporation into multilamellar lipid micelles, although a proportion of absorbed β-carotene is converted to retinol within intestinal mucosal cells. Unaltered β-carotene is transported via the lymph to the plasma where it is associated with lipoproteins. Tissue uptake and distribution are not well characterized. When the intake of β-carotene is consistently high, long-term accumulation occurs preferentially in adipose tissues.

Experiments in rats have shown that the conversion of β-carotene to retinol is regulated by the levels of β-carotene and of preformed vitamin A. *In vitro* studies have shown that other β-carotene derivatives may also occur, but their biological activity, and whether they are synthesized in vivo, is unknown. Carotenoid absorption and metabolism vary considerably between animal species (Lee and others 1999). No single species is a good model for studying biokinetics and metabolism of β-carotene in humans. The rat is unsuitable because it is highly efficient at converting β-carotene to vitamin A, and significant levels of unaltered β-carotene are absorbed only when very high doses are given for a prolonged time. The preruminant calf, the ferret, and the Mongolian gerbil are more useful models, although there are many differences in carotenoid absorption, distribution, and metabolism between these animals and humans.

Toxicity of Carotene

Dietary β-carotene has not been shown to be toxic in humans (Expert Group on Vitamins and Minerals 2003). Hypercarotenaemia (high plasma β-carotene) has not been associated with adverse effects other than reversible yellowing of the skin, known as hypercarotenodermia. Long-term oral β-carotene therapy in doses up to 300 μg/d showed no toxic effects in individuals with erythropoietic protoporphyria. Vitamin A toxicity does not occur because the metabolic conversion is regulated by vitamin A status. Reproductive toxicity or teratogenicity associated with high β-carotene intake, either before or during pregnancy, has not been reported. Two large-scale supplementation trials testing the hypothesis that β-carotene supplementation in smokers would reduce the incidence of cancer have shown an association of high dose β-carotene supplementation (20 to 30 mg/d) with increased incidence of lung cancer in smokers and asbestos-exposed individuals (Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn and others 1996). No statistically significant differences in other cancer types were observed in these studies.

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Marasmus is the predominant form of PEM in most developing countries and can affect all age groups. Kwashiorkor is less common and is usually manifested as marasmic–kwashiorkor, especially in young children. It tends to occur where the staple foods are low in protein and excessively starchy (for example, yam [Dioscorea], cassava, sweetpotato, taro, plantain, and banana).

The assessment of PEM uses indices derived from measurements of body stature or length, weight, age, and presence or absence of edema that are compared with a reference population considered to have normal growth characteristics---usually the United States, although this has been questioned. The indices are underweight (low weight for age), stunting (low height for age), and wasting (low weight for height). National and regional level data on nutritional status have been compiled systematically for more than 20 y by the WHO Global Database on Child Growth and Malnutrition, which represents most of the children worldwide (de Onis and others 2004). Because the population in Asia is larger than elsewhere in the world, the number of undernourished is greatest in Asia, followed by Africa, although the proportion that are undernourished is greatest in Africa (de Onis and others 2004).

Views on the causes of PEM have evolved over time. By the 1960s, for example, it was known that the supply of protein in cereal-based diets was adequate (Hegsted and others 1946). The FAO nevertheless considered protein deficiency to be serious and widespread in developing countries. This was based on the observations that kwashiorkor, which was common among 1- to 4-y-old children in developing countries, (1) responded to therapy that included relatively high-protein supplements and (2) caused liver damage. Because liver cirrhosis was common among adults in Africa, the FAO thought this too indicated that African diets remained protein-deficient throughout life and the same might also be true in all developing countries. Milk and milk powder were expensive and in short supply. Consequently, much work was carried out to develop and test economical alternatives to milk powder based on locally available cereals and oilseed flours, fish protein concentrate, and single-cell protein from fermenting microorganisms. The work ended once it was realized that most children with kwashiorkor had been raised on diets that were as deficient in energy as they were in protein. The diets were also too bulky, which meant children could only eat a limited and inadequate amount (Whitehead 1973). Consensus was reached that the solution was to provide more high-energy foods and correct electrolyte deficiencies rather than concentrate just on protein (Waterlow 1961; McLaren 1974; Carpenter 1994), although it was recognized that diets based on staples such as cassava, bananas, and yams (Dioscorea) that are both very bulky and in low in protein present special challenges (Nicol 1971). In addition, initial treatment for severe PEM requires comparatively low levels of protein with a high biological value to ensure optimal utilization of protein for tissue maintenance and to prevent overloading the impaired metabolic system with superfluous amino acids and their nitrogenous metabolites. Following this initial treatment, high-protein diets such as milk-based formula can be used to stimulate growth and recovery (for example, Müller and Krawinkel 2005).

The importance of good nutrition starts at conception because fetal growth and development lay the foundation for health at all later ages. A fetus depends on the mother's ability to deliver nutrients, and this is greatly influenced by her nutritional state at the time she becomes pregnant as well as throughout pregnancy. Poor fetal growth and development have major consequences for the offspring, and these also have implications for health in the neonatal period, infancy, childhood, adolescence, and later life. Undernutrition in early childhood has serious consequences as these children are not only more prone to illness, but they also tend to have more severe illnesses than well-nourished children. Undernourished children are also at greater risk of dying. Undernutrition in school age children, much of which probably started in early childhood, adversely affects school attendance, performance, and learning. A stunted girl is more likely to become a stunted adolescent and later a stunted woman. Apart from direct effects on her health and productivity, undernutrition in an adult woman increases the chance that her children will be born with low birth weight, and the cycle begins again (United Nations Standing Committee on Nutrition 2004). Interventions to control undernutrition are therefore needed at different stages in the life cycle. These include improved child care and feeding practices for infants and young children; the provision of specific micronutrients through food fortification, biofortification, and pharmaceutical supplementation; improved access to health care; household

food security; good hygiene and sanitation; and universal female education.

The WHO global strategy for feeding infants and young children highlights the importance of good feeding practices during the transition period between exclusive breastfeeding and full weaning, when breastfed children also need complementary food. In addition to being safe and timely, the diet must be adequate—defined as providing sufficient energy, protein, and micronutrients to meet a growing child's nutritional needs (WHO 2002). Children need a variety of foods to ensure that nutrient needs are met. Diets that lack animal source foods (meat, poultry, fish or eggs, plus milk products) cannot meet the nutrient requirements for children ages 6 to 24 mo unless fortified foods or supplements are used. If milk and other animal source foods are not taken in adequate amounts, both grains and legumes should be consumed daily, preferably within the same meal, to ensure adequate protein quality (WHO 2004). In addition to the availability of these foods, their accessibility to the population depending on, for example, infrastructure and economic resources needs to be considered.

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Appendix 5: Deregulation (Excerpted from Al-Babili and Beyer 2005)

Deregulation is one term used for the process that eventually leads to the registration of a product and the granting of its unlimited commercial or noncommercial use. The national authorities of the respective countries in which the product is intended for use govern deregulation of GMO plants and the foods derived from these. The process is driven by a science-based risk assessment of the intended product and the environments into which it will be released. Current data requirements can be extensive and are comprehensive, and require the submission of publication quality data reports. The development of these materials can be time- consuming and costly. The studies required to compile a regulatory dossier fall into 2 categories: event-dependent and event-independent studies.

Event-independent studies can include biochemical analysis (function, specificity and mode of action), studies on toxicity and the allergenic potential of the newly expressed proteins. In addition, dietary exposure modeling and bioavailability studies can be conducted with the newly expressed proteins and, in the case of enzymes, with the products produced by catalysis.

Event-dependent studies vary by country and can encompass the characterization of the quality of integration, such as copy number, the determination of the site of integration in the host genome, demonstration of the absence of gene disruption, completeness of integrated DNA sequences, and the presence of the marker gene at the same locus and absence of vector DNA sequences. Phenotypic and biochemical evidence for trait stability, including the monitoring of gene expression levels at key growth stages and Mendelian inheritance over several generations, are usually components of these studies. Field performance of typical agronomic traits, such as yield, and pest and disease resistance, is an additional issue. The assessment is also based on the comparison of the compositional analyses from materials harvested at different locations with the equivalent nontransgenic crop (and food) comparator. Event-dependent regulatory data are usually not gathered until the developer is satisfied with the technical performance of the product and a few events (usually one) have been chosen. Assessment of the potential risks of GM crops is on a case-by-case basis within a scientific framework. A GMO crop producing β-carotene must be viewed differently than a crop that produces an insecticide. Similarly, GR events expressing a phytoene synthase from maize, a widely consumed crop, might be viewed differently than one using the enzyme from a nonfood plant, such as daffodil.

From a scientific point of view it is often hard to understand why randomly mutagenized crop plants can enter into breeding lines easily, whereas GMOs, with considerably fewer genetic modifications, have a much more rigorous assessment. To have rational, science-based regulatory requirements it would be necessary to realize the benefits (not only potential risks) that GMO crops can provide (see lecture by Ingo Potrykus at http://www.syngentafoundation.com/golden_rice/index.htm). It is unhelpful to raise the regulatory requirements to the point where anything that appears technically feasible is being requested or is being offered to be applied (Kuiper and others

2001), with the only justification that the genetically modified organism involved is little understood. This strategy can raise costs to unaffordable levels. We do not understand bred varieties with their complex genomic changes much better at the molecular level, but we tend to consider the traditional way of producing new varieties safe in contrast to the novel way of producing GMOs even though this is not based on any rational evaluation. It is the final product and not the technology used to produce it that should be scrutinized.

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Appendix 1: Vitamin A Deficiency: A Global Risk

Vitamin A deficiency (VAD) is a global public health challenge, particularly in infants, children, and pregnant and lactating women because of the extra requirements to support fetal, infant, and child growth (Figure A1-1). Deficiency can result in reduced resistance to infection, impaired cellular differentiation, xerophthalmia, and ultimately blindness and death; it is also associated with anemia. More than 100 million children and as many as 7 million pregnant women living in over 100 countries have VAD (West 2002; WHO 2003). It is estimated that between 1.3 and 2.5 million deaths of children aged 6 mo to 5 y could be prevented each year by proper vitamin A nutrition (Figure A1-2). Two million children have clinical or severe visual problems and more than 250000 children become blind as a result of VAD each year, one-half of whom die within a year of losing their sight. (FAO 2002). The chances of death from measles or diarrheal disease are greatly increased by VAD (FAO/WHO 2002). VAD also interacts with other nutrients; for example, iron metabolism is negatively affected and iron is not incorporated effectively into hemoglobin (Hodges and others 1978).

Many countries with a high prevalence of VAD rely on rice as a major source of energy. Rice does not contain β-carotene, the direct precursor of vitamin A. Attempts to provide biannual vitamin A supplements to all children less than 6 y of age have been difficult to sustain and are comparatively expensive (Bouis and others 2003). More recently, fortification of foods such as sugar and cereal flours with vitamin A has come to the fore. Lack of dietary diversity causes VAD, and historically, emphasis has been on increasing the intake of green leafy vegetables and yellow-orange fruits and vegetables to improve vitamin A intake. However, the β-carotene in green leafy vegetable is less well absorbed than that from yellow-orange fruits and roots or tubers, although the method of preparation, including the presence of dietary lipid, can improve bioefficacy (Yeum and Russell 2002; Haskell and others 2004). The Inst. of Medicine (2001) has proposed that 12 μg of β-carotene are required to provide 1 μg of vitamin A, while a conversion factor of 1 to 24 μg has been set for other provitamin A carotenoids, that is, β-cryptoxanthin and α-carotene.

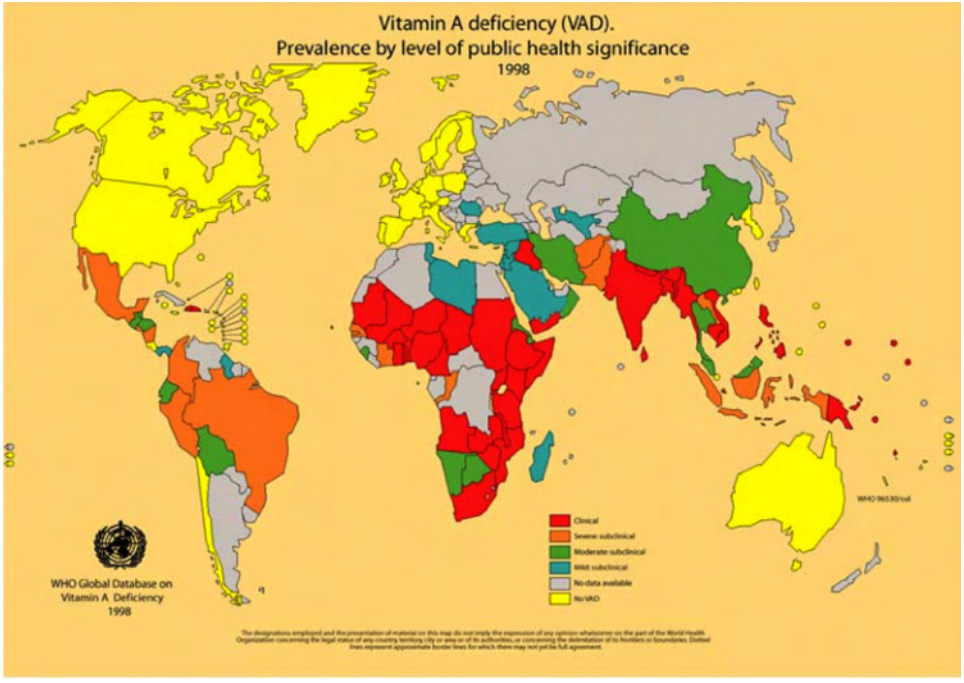

Figure A1-1—Vitamin A deficiency around the globe (FAO/WHO 2002).

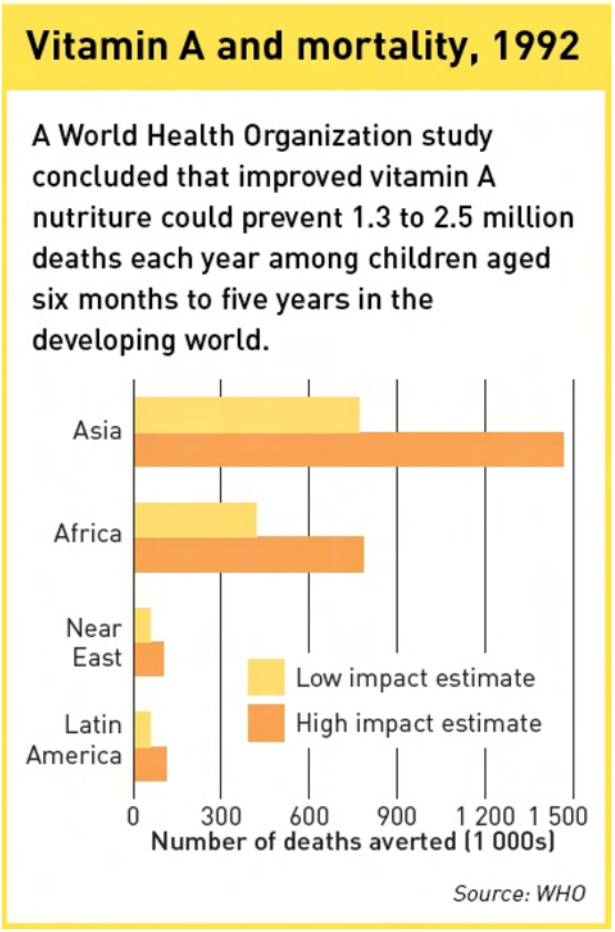

Figure A1-2—WHO estimates of childhood mortality (FAO 1992).

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Appendix 2: Biosynthesis of β-Carotene

The details of the biosynthesis of β-carotene and other carotenoids in plants have been reviewed elsewhere (Cunningham and Gantt 1998; Sandmann 2001; Taylor and Ramsay 2005). The initial steps in the biosynthesis of β-carotene involve intermediates that are also precursors to other products in plant cells. For example, the 5-carbon molecule (C5) isopentenyl pyrophosphate (IPP) is the building unit for the synthesis of various kinds of terpenoids, including β-carotenes, plant hormones, and other secondary metabolites and defense molecules. IPP is formed both in the cytosol, through the mevalonate pathway, and in plastids, through the deoxy-xylulose-phosphate pathway. In plastids, IPP serves as substrate for carotenoid biosynthesis, among others, and in the cytosol for some plant hormones and steroids.

Because IPP isomerase is present in plant plastids and its activity is the limiting step of carotenoid synthesis in etioplasts, it has been concluded that IPP is the major or only product of the 1-deoxyxylulose-5-phosphate pathway–-the reaction sequence leading to cyclic carotenoids (Albrecht and others 1994).

The 1st steps in the biosynthesis from IPP to β-carotene entail the formation of the C20 compound geranylgeranylphosphate (GGPP) by condensation of 4 C5-precursors in total. First, 1 IPP molecule is isomerized by the enzyme IPP isomerase into dimethylallyl-pyrophosphate (DMAPP). This activated form of IPP then reacts with 3 IPP molecules in 3 discrete reactions, leading to the formation of GGPP catalyzed by the enzyme GGPP synthase. Two molecules of GGPP are subsequently condensed to phytoene (C40) under the influence of the enzyme phytoene synthase.

Phytoene enters a series of dehydrogenation and ring formation reactions, resulting in the formation of β-carotene. Phytoene desaturase (PDS) and zeta carotene desaturase (ZDS)

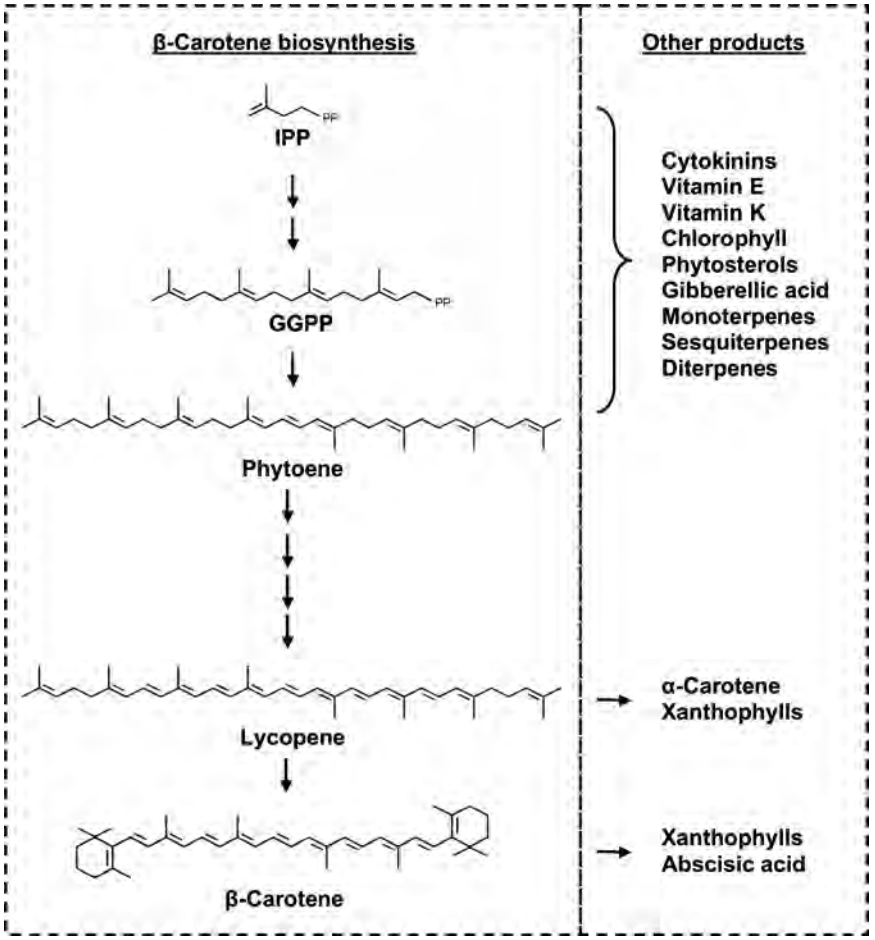

Figure A2-1—Biosynthesis of β-carotene in plants and other products originating from intermediates in the same pathway. [Based on "Biochemical Pathways" chart of Roche Applied Sciences (www. expasy.org/ tools/pathways/) and Taylor and Ramsay (2005)].

catalyze 4 dehydrogenation reactions, while carotenoid isomerase (CRTISO) catalyzes the *cis-trans* isomerization of cis double bonds, particularly the 15-*cis*-double bond between carbon atoms, so that ultimately all-*trans*-lycopene is formed. The enzyme lycopene β-cyclase (LCYB) converts lycopene into β-carotene through 2 cyclization reactions involving the formation of C6-rings at both ends of the molecule. In addition, β-carotene itself may be converted to xanthophylls, which are carotenoid molecules with hydroxylated rings. Further reactions of xanthophylls lead to, for example, formation of the plant growth regulator, abscisic acid (Cunningham and Gantt 1998; Taylor and Ramsay 2005).

Figure A2-1 provides a schematic overview of the pathway leading to biosynthesis of β-carotene and products derived from branches of the same pathway. Increasing the flux through this pathway toward β-carotene or intermediates may alter the biosynthesis of other metabolites. For example, Fray and others (1995) found that levels of intermediates, including phytoene, lycopene, and ζ-carotene, had increased in tomato plants expressing phytoene synthase. In addition, transgenic plants showed stunted growth due to decreased production of gibberellic acid-based plant growth regulators. Chlorophyll biosynthesis was decreased in these plants as well.

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Appendix 3: Physiology of β-Carotene

Intake of β-Carotene

Beta-carotene is an important provitamin A carotenoid, that is, precursor of vitamin A, but is not an essential nutrient and has no dietary reference value. Dietary intakes are expressed as total micrograms of retinol equivalents (RE), in which 12 μg β-carotene is equivalent nutritionally to 1 μg RE. The FAO/WHO (2002) recommended safe dietary intake of vitamin A is 375 μg/d for infants, 400 to 450 μg/d for children 1 to 6 y, 800 μg/d for pregnant women, and 850 μg/d for lactating women.

Provitamin A carotenoids are generally ingested from plant foods and can be biologically transformed to vitamin A. Globally, about 60% of dietary vitamin A comes from provitamins A. Many factors influence the absorption and utilization of provitamin A, such as the vitamin A status of the individual; the amount, type, and physical form of the carotenoids in the diet; the intake of fat, vitamin A, and fiber; the individual's protein and zinc status; and the presence of certain diseases and parasitic infections (de Pee and West 1996). Between 10% and 90% of all dietary β-carotene is absorbed, and absorption decreases as intake increases. Bioavailability is reduced in very low-fat diets.

Metabolism of β-Carotene

The metabolism of β-carotene in humans and animals is well documented, with excellent reviews by Olson (1996), Parker (1997), Burri and Clifford (2004), and Bendich (2004).

In humans, dietary fat and bile salts facilitate the absorption of β-carotene that occurs in the upper small intestine. Absorption occurs via incorporation into multilamellar lipid micelles, although a proportion of absorbed β-carotene is converted to retinol within intestinal mucosal cells. Unaltered β-carotene is transported via the lymph to the plasma where it is associated with lipoproteins. Tissue uptake and distribution are not well characterized. When the intake of β-carotene is consistently high, long-term accumulation occurs preferentially in adipose tissues.

Experiments in rats have shown that the conversion of β-carotene to retinol is regulated by the levels of β-carotene and of preformed vitamin A. *In vitro* studies have shown that other β-carotene derivatives may also occur, but their biological activity, and whether they are synthesized in vivo, is unknown. Carotenoid absorption and metabolism vary considerably between animal species (Lee and others 1999). No single species is a good model for studying biokinetics and metabolism of β-carotene in humans. The rat is unsuitable because it is highly efficient at converting β-carotene to vitamin A, and significant levels of unaltered β-carotene are absorbed only when very high doses are given for a prolonged time. The preruminant calf, the ferret, and the Mongolian gerbil are more useful models, although there are many differences in carotenoid absorption, distribution, and metabolism between these animals and humans.

Toxicity of Carotene

Dietary β-carotene has not been shown to be toxic in humans (Expert Group on Vitamins and Minerals 2003). Hypercarotenaemia (high plasma β-carotene) has not been associated with adverse effects other than reversible yellowing of the skin, known as hypercarotenodermia. Long-term oral β-carotene therapy in doses up to 300 μg/d showed no toxic effects in individuals with erythropoietic protoporphyria. Vitamin A toxicity does not occur because the metabolic conversion is regulated by vitamin A status. Reproductive toxicity or teratogenicity associated with high β-carotene intake, either before or during pregnancy, has not been reported. Two large-scale supplementation trials testing the hypothesis that β-carotene supplementation in smokers would reduce the incidence of cancer have shown an association of high dose β-carotene supplementation (20 to 30 mg/d) with increased incidence of lung cancer in smokers and asbestos-exposed individuals (Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn and others 1996). No statistically significant differences in other cancer types were observed in these studies.

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Appendix 4: Protein-Energy Malnutrition

Malnutrition includes both under- and overnutrition. Undernutrition is the general term for an inadequate intake of energy or specific vitamins and minerals that are collectively known as micronutrients. Overnutrition is the opposite. This section focuses on undernutrition, specifically protein-energy malnutrition (PEM). PEM syndrome results from inadequate food intake and is characterized by energy deficits, due to a reduced intake of all macronutrients and often many micronutrients as well. Clinically, PEM manifests as emaciation (marasmus), edema (kwashiorkor), or a combination of the two (marasmic–kwashiorkor). The form depends on the balance of nonprotein and protein sources of energy. Marasmus occurs when energy intake is insufficient to meet requirements and the body is forced to draw on its own stores: first glycogen, then skeletal muscle and triglycerides in fat depots. Consequently, the individual becomes thin from loss of muscle and body fat. In kwashiorkor, dietary protein deficiency is usually more marked than the energy deficiency, and this results in decreased protein synthesis and edema. Children with marasmic–kwashiorkor have some edema and more body fat than those with marasmus.

Marasmus is the predominant form of PEM in most developing countries and can affect all age groups. Kwashiorkor is less common and is usually manifested as marasmic–kwashiorkor, especially in young children. It tends to occur where the staple foods are low in protein and excessively starchy (for example, yam [Dioscorea], cassava, sweetpotato, taro, plantain, and banana).

The assessment of PEM uses indices derived from measurements of body stature or length, weight, age, and presence or absence of edema that are compared with a reference population considered to have normal growth characteristics---usually the United States, although this has been questioned. The indices are underweight (low weight for age), stunting (low height for age), and wasting (low weight for height). National and regional level data on nutritional status have been compiled systematically for more than 20 y by the WHO Global Database on Child Growth and Malnutrition, which represents most of the children worldwide (de Onis and others 2004). Because the population in Asia is larger than elsewhere in the world, the number of undernourished is greatest in Asia, followed by Africa, although the proportion that are undernourished is greatest in Africa (de Onis and others 2004).

Views on the causes of PEM have evolved over time. By the 1960s, for example, it was known that the supply of protein in cereal-based diets was adequate (Hegsted and others 1946). The FAO nevertheless considered protein deficiency to be serious and widespread in developing countries. This was based on the observations that kwashiorkor, which was common among 1- to 4-y-old children in developing countries, (1) responded to therapy that included relatively high-protein supplements and (2) caused liver damage. Because liver cirrhosis was common among adults in Africa, the FAO thought this too indicated that African diets remained protein-deficient throughout life and the same might also be true in all developing countries. Milk and milk powder were expensive and in short supply. Consequently, much work was carried out to develop and test economical alternatives to milk powder based on locally available cereals and oilseed flours, fish protein concentrate, and single-cell protein from fermenting microorganisms. The work ended once it was realized that most children with kwashiorkor had been raised on diets that were as deficient in energy as they were in protein. The diets were also too bulky, which meant children could only eat a limited and inadequate amount (Whitehead 1973). Consensus was reached that the solution was to provide more high-energy foods and correct electrolyte deficiencies rather than concentrate just on protein (Waterlow 1961; McLaren 1974; Carpenter 1994), although it was recognized that diets based on staples such as cassava, bananas, and yams (Dioscorea) that are both very bulky and in low in protein present special challenges (Nicol 1971). In addition, initial treatment for severe PEM requires comparatively low levels of protein with a high biological value to ensure optimal utilization of protein for tissue maintenance and to prevent overloading the impaired metabolic system with superfluous amino acids and their nitrogenous metabolites. Following this initial treatment, high-protein diets such as milk-based formula can be used to stimulate growth and recovery (for example, Müller and Krawinkel 2005).

The importance of good nutrition starts at conception because fetal growth and development lay the foundation for health at all later ages. A fetus depends on the mother's ability to deliver nutrients, and this is greatly influenced by her nutritional state at the time she becomes pregnant as well as throughout pregnancy. Poor fetal growth and development have major consequences for the offspring, and these also have implications for health in the neonatal period, infancy, childhood, adolescence, and later life. Undernutrition in early childhood has serious consequences as these children are not only more prone to illness, but they also tend to have more severe illnesses than well-nourished children. Undernourished children are also at greater risk of dying. Undernutrition in school age children, much of which probably started in early childhood, adversely affects school attendance, performance, and learning. A stunted girl is more likely to become a stunted adolescent and later a stunted woman. Apart from direct effects on her health and productivity, undernutrition in an adult woman increases the chance that her children will be born with low birth weight, and the cycle begins again (United Nations Standing Committee on Nutrition 2004). Interventions to control undernutrition are therefore needed at different stages in the life cycle. These include improved child care and feeding practices for infants and young children; the provision of specific micronutrients through food fortification, biofortification, and pharmaceutical supplementation; improved access to health care; household

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Appendix 5: Deregulation (Excerpted from Al-Babili and Beyer 2005)

Deregulation is one term used for the process that eventually leads to the registration of a product and the granting of its unlimited commercial or noncommercial use. The national authorities of the respective countries in which the product is intended for use govern deregulation of GMO plants and the foods derived from these. The process is driven by a science-based risk assessment of the intended product and the environments into which it will be released. Current data requirements can be extensive and are comprehensive, and require the submission of publication quality data reports. The development of these materials can be time- consuming and costly. The studies required to compile a regulatory dossier fall into 2 categories: event-dependent and event-independent studies.

Event-independent studies can include biochemical analysis (function, specificity and mode of action), studies on toxicity and the allergenic potential of the newly expressed proteins. In addition, dietary exposure modeling and bioavailability studies can be conducted with the newly expressed proteins and, in the case of enzymes, with the products produced by catalysis.

Event-dependent studies vary by country and can encompass the characterization of the quality of integration, such as copy number, the determination of the site of integration in the host genome, demonstration of the absence of gene disruption, completeness of integrated DNA sequences, and the presence of the marker gene at the same locus and absence of vector DNA sequences. Phenotypic and biochemical evidence for trait stability, including the monitoring of gene expression levels at key growth stages and Mendelian inheritance over several generations, are usually components of these studies. Field performance of typical agronomic traits, such as yield, and pest and disease resistance, is an additional issue. The assessment is also based on the comparison of the compositional analyses from materials harvested at different locations with the equivalent nontransgenic crop (and food) comparator. Event-dependent regulatory data are usually not gathered until the developer is satisfied with the technical performance of the product and a few events (usually one) have been chosen. Assessment of the potential risks of GM crops is on a case-by-case basis within a scientific framework. A GMO crop producing β-carotene must be viewed differently than a crop that produces an insecticide. Similarly, GR events expressing a phytoene synthase from maize, a widely consumed crop, might be viewed differently than one using the enzyme from a nonfood plant, such as daffodil.

From a scientific point of view it is often hard to understand why randomly mutagenized crop plants can enter into breeding lines easily, whereas GMOs, with considerably fewer genetic modifications, have a much more rigorous assessment. To have rational, science-based regulatory requirements it would be necessary to realize the benefits (not only potential risks) that GMO crops can provide (see lecture by Ingo Potrykus at http://www.syngentafoundation.com/golden_rice/index.htm). It is unhelpful to raise the regulatory requirements to the point where anything that appears technically feasible is being requested or is being offered to be applied (Kuiper and others

2001), with the only justification that the genetically modified organism involved is little understood. This strategy can raise costs to unaffordable levels. We do not understand bred varieties with their complex genomic changes much better at the molecular level, but we tend to consider the traditional way of producing new varieties safe in contrast to the novel way of producing GMOs even though this is not based on any rational evaluation. It is the final product and not the technology used to produce it that should be scrutinized.

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Appendix 1: Vitamin A Deficiency: A Global Risk

Vitamin A deficiency (VAD) is a global public health challenge, particularly in infants, children, and pregnant and lactating women because of the extra requirements to support fetal, infant, and child growth (Figure A1-1). Deficiency can result in reduced resistance to infection, impaired cellular differentiation, xerophthalmia, and ultimately blindness and death; it is also associated with anemia. More than 100 million children and as many as 7 million pregnant women living in over 100 countries have VAD (West 2002; WHO 2003). It is estimated that between 1.3 and 2.5 million deaths of children aged 6 mo to 5 y could be prevented each year by proper vitamin A nutrition (Figure A1-2). Two million children have clinical or severe visual problems and more than 250000 children become blind as a result of VAD each year, one-half of whom die within a year of losing their sight. (FAO 2002). The chances of death from measles or diarrheal disease are greatly increased by VAD (FAO/WHO 2002). VAD also interacts with other nutrients; for example, iron metabolism is negatively affected and iron is not incorporated effectively into hemoglobin (Hodges and others 1978).

Many countries with a high prevalence of VAD rely on rice as a major source of energy. Rice does not contain β-carotene, the direct precursor of vitamin A. Attempts to provide biannual vitamin A supplements to all children less than 6 y of age have been difficult to sustain and are comparatively expensive (Bouis and others 2003). More recently, fortification of foods such as sugar and cereal flours with vitamin A has come to the fore. Lack of dietary diversity causes VAD, and historically, emphasis has been on increasing the intake of green leafy vegetables and yellow-orange fruits and vegetables to improve vitamin A intake. However, the β-carotene in green leafy vegetable is less well absorbed than that from yellow-orange fruits and roots or tubers, although the method of preparation, including the presence of dietary lipid, can improve bioefficacy (Yeum and Russell 2002; Haskell and others 2004). The Inst. of Medicine (2001) has proposed that 12 μg of β-carotene are required to provide 1 μg of vitamin A, while a conversion factor of 1 to 24 μg has been set for other provitamin A carotenoids, that is, β-cryptoxanthin and α-carotene.

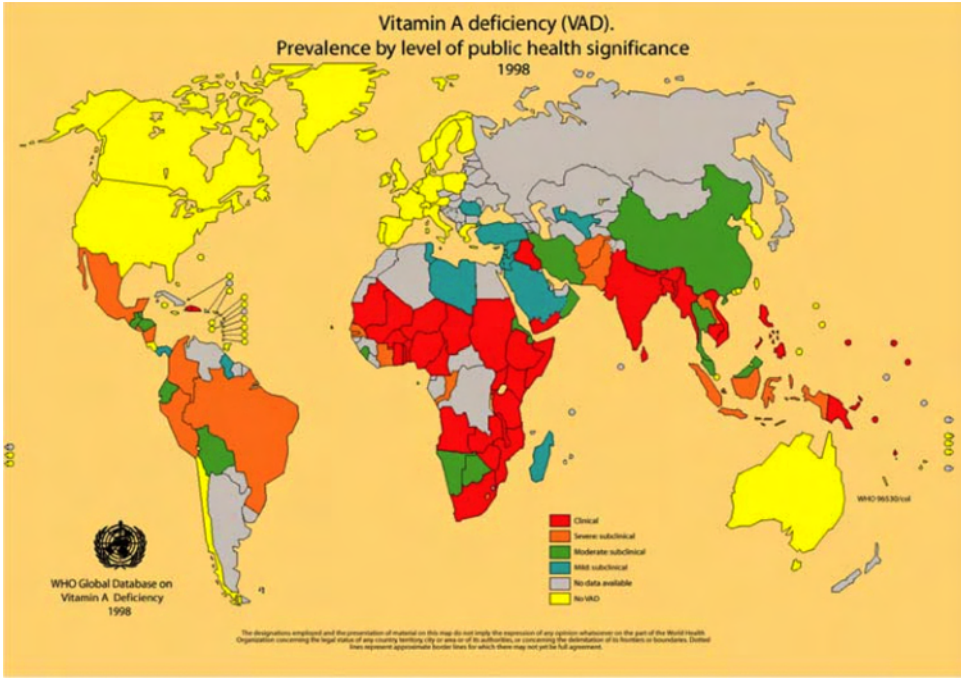

Figure A1-1—Vitamin A deficiency around the globe (FAO/WHO 2002).

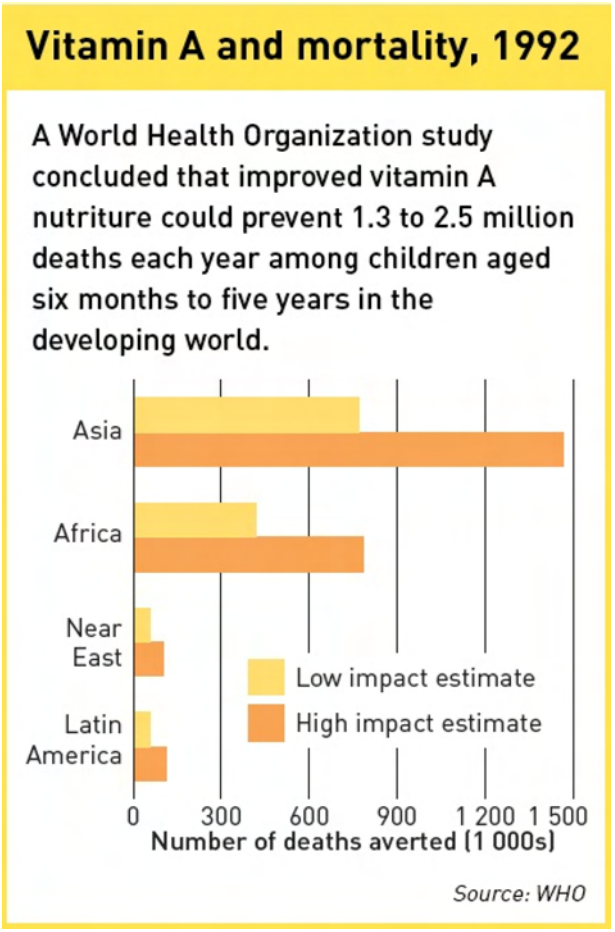

Figure A1-2—WHO estimates of childhood mortality (FAO 1992).

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Appendix 2: Biosynthesis of β-Carotene

The details of the biosynthesis of β-carotene and other carotenoids in plants have been reviewed elsewhere (Cunningham and Gantt 1998; Sandmann 2001; Taylor and Ramsay 2005). The initial steps in the biosynthesis of β-carotene involve intermediates that are also precursors to other products in plant cells. For example, the 5-carbon molecule (C5) isopentenyl pyrophosphate (IPP) is the building unit for the synthesis of various kinds of terpenoids, including β-carotenes, plant hormones, and other secondary metabolites and defense molecules. IPP is formed both in the cytosol, through the mevalonate pathway, and in plastids, through the deoxy-xylulose-phosphate pathway. In plastids, IPP serves as substrate for carotenoid biosynthesis, among others, and in the cytosol for some plant hormones and steroids.

Because IPP isomerase is present in plant plastids and its activity is the limiting step of carotenoid synthesis in etioplasts, it has been concluded that IPP is the major or only product of the 1-deoxyxylulose-5-phosphate pathway–-the reaction sequence leading to cyclic carotenoids (Albrecht and others 1994).

The 1st steps in the biosynthesis from IPP to β-carotene entail the formation of the C20 compound geranylgeranylphosphate (GGPP) by condensation of 4 C5-precursors in total. First, 1 IPP molecule is isomerized by the enzyme IPP isomerase into dimethylallyl-pyrophosphate (DMAPP). This activated form of IPP then reacts with 3 IPP molecules in 3 discrete reactions, leading to the formation of GGPP catalyzed by the enzyme GGPP synthase. Two molecules of GGPP are subsequently condensed to phytoene (C40) under the influence of the enzyme phytoene synthase.

Phytoene enters a series of dehydrogenation and ring formation reactions, resulting in the formation of β-carotene. Phytoene desaturase (PDS) and zeta carotene desaturase (ZDS)

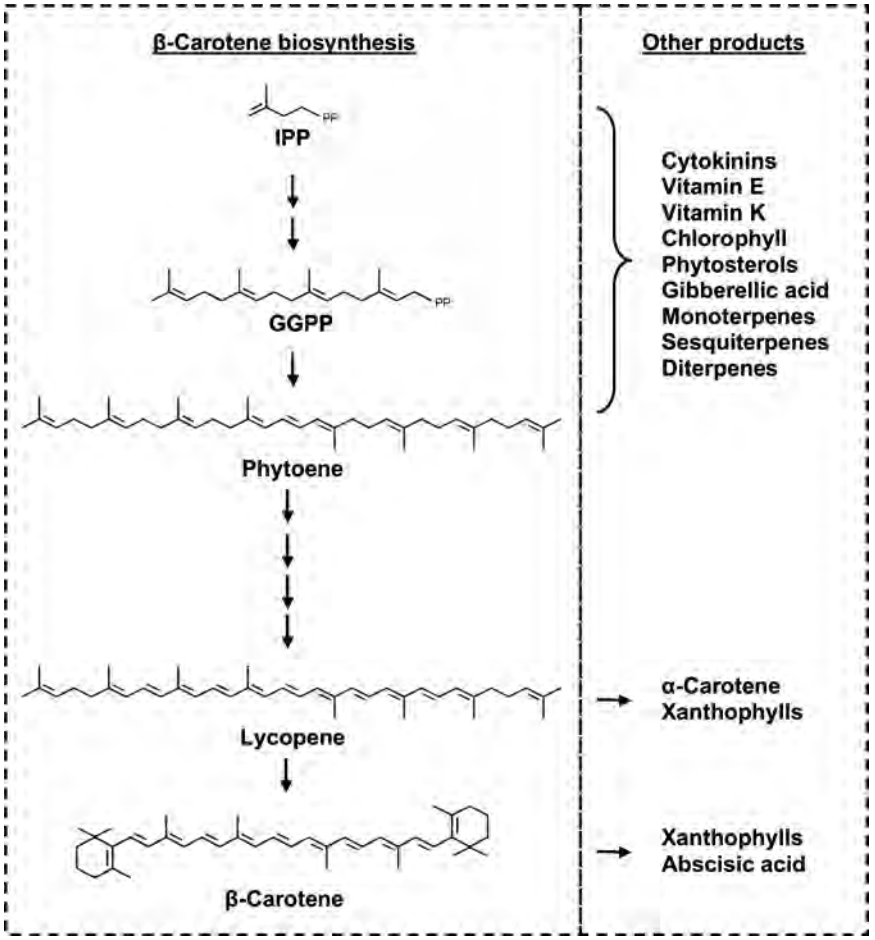

Figure A2-1—Biosynthesis of β-carotene in plants and other products originating from intermediates in the same pathway. [Based on "Biochemical Pathways" chart of Roche Applied Sciences (www. expasy.org/ tools/pathways/) and Taylor and Ramsay (2005)].

catalyze 4 dehydrogenation reactions, while carotenoid isomerase (CRTISO) catalyzes the *cis-trans* isomerization of cis double bonds, particularly the 15-*cis*-double bond between carbon atoms, so that ultimately all-*trans*-lycopene is formed. The enzyme lycopene β-cyclase (LCYB) converts lycopene into β-carotene through 2 cyclization reactions involving the formation of C6-rings at both ends of the molecule. In addition, β-carotene itself may be converted to xanthophylls, which are carotenoid molecules with hydroxylated rings. Further reactions of xanthophylls lead to, for example, formation of the plant growth regulator, abscisic acid (Cunningham and Gantt 1998; Taylor and Ramsay 2005).

Figure A2-1 provides a schematic overview of the pathway leading to biosynthesis of β-carotene and products derived from branches of the same pathway. Increasing the flux through this pathway toward β-carotene or intermediates may alter the biosynthesis of other metabolites. For example, Fray and others (1995) found that levels of intermediates, including phytoene, lycopene, and ζ-carotene, had increased in tomato plants expressing phytoene synthase. In addition, transgenic plants showed stunted growth due to decreased production of gibberellic acid-based plant growth regulators. Chlorophyll biosynthesis was decreased in these plants as well.

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Appendix 3: Physiology of β-Carotene

Intake of β-Carotene

Beta-carotene is an important provitamin A carotenoid, that is, precursor of vitamin A, but is not an essential nutrient and has no dietary reference value. Dietary intakes are expressed as total micrograms of retinol equivalents (RE), in which 12 μg β-carotene is equivalent nutritionally to 1 μg RE. The FAO/WHO (2002) recommended safe dietary intake of vitamin A is 375 μg/d for infants, 400 to 450 μg/d for children 1 to 6 y, 800 μg/d for pregnant women, and 850 μg/d for lactating women.

Provitamin A carotenoids are generally ingested from plant foods and can be biologically transformed to vitamin A. Globally, about 60% of dietary vitamin A comes from provitamins A. Many factors influence the absorption and utilization of provitamin A, such as the vitamin A status of the individual; the amount, type, and physical form of the carotenoids in the diet; the intake of fat, vitamin A, and fiber; the individual's protein and zinc status; and the presence of certain diseases and parasitic infections (de Pee and West 1996). Between 10% and 90% of all dietary β-carotene is absorbed, and absorption decreases as intake increases. Bioavailability is reduced in very low-fat diets.

Metabolism of β-Carotene

The metabolism of β-carotene in humans and animals is well documented, with excellent reviews by Olson (1996), Parker (1997), Burri and Clifford (2004), and Bendich (2004).

In humans, dietary fat and bile salts facilitate the absorption of β-carotene that occurs in the upper small intestine. Absorption occurs via incorporation into multilamellar lipid micelles, although a proportion of absorbed β-carotene is converted to retinol within intestinal mucosal cells. Unaltered β-carotene is transported via the lymph to the plasma where it is associated with lipoproteins. Tissue uptake and distribution are not well characterized. When the intake of β-carotene is consistently high, long-term accumulation occurs preferentially in adipose tissues.

Experiments in rats have shown that the conversion of β-carotene to retinol is regulated by the levels of β-carotene and of preformed vitamin A. *In vitro* studies have shown that other β-carotene derivatives may also occur, but their biological activity, and whether they are synthesized in vivo, is unknown. Carotenoid absorption and metabolism vary considerably between animal species (Lee and others 1999). No single species is a good model for studying biokinetics and metabolism of β-carotene in humans. The rat is unsuitable because it is highly efficient at converting β-carotene to vitamin A, and significant levels of unaltered β-carotene are absorbed only when very high doses are given for a prolonged time. The preruminant calf, the ferret, and the Mongolian gerbil are more useful models, although there are many differences in carotenoid absorption, distribution, and metabolism between these animals and humans.

Toxicity of Carotene

Dietary β-carotene has not been shown to be toxic in humans (Expert Group on Vitamins and Minerals 2003). Hypercarotenaemia (high plasma β-carotene) has not been associated with adverse effects other than reversible yellowing of the skin, known as hypercarotenodermia. Long-term oral β-carotene therapy in doses up to 300 μg/d showed no toxic effects in individuals with erythropoietic protoporphyria. Vitamin A toxicity does not occur because the metabolic conversion is regulated by vitamin A status. Reproductive toxicity or teratogenicity associated with high β-carotene intake, either before or during pregnancy, has not been reported. Two large-scale supplementation trials testing the hypothesis that β-carotene supplementation in smokers would reduce the incidence of cancer have shown an association of high dose β-carotene supplementation (20 to 30 mg/d) with increased incidence of lung cancer in smokers and asbestos-exposed individuals (Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994; Omenn and others 1996). No statistically significant differences in other cancer types were observed in these studies.

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Appendix 4: Protein-Energy Malnutrition

Malnutrition includes both under- and overnutrition. Undernutrition is the general term for an inadequate intake of energy or specific vitamins and minerals that are collectively known as micronutrients. Overnutrition is the opposite. This section focuses on undernutrition, specifically protein-energy malnutrition (PEM). PEM syndrome results from inadequate food intake and is characterized by energy deficits, due to a reduced intake of all macronutrients and often many micronutrients as well. Clinically, PEM manifests as emaciation (marasmus), edema (kwashiorkor), or a combination of the two (marasmic–kwashiorkor). The form depends on the balance of nonprotein and protein sources of energy. Marasmus occurs when energy intake is insufficient to meet requirements and the body is forced to draw on its own stores: first glycogen, then skeletal muscle and triglycerides in fat depots. Consequently, the individual becomes thin from loss of muscle and body fat. In kwashiorkor, dietary protein deficiency is usually more marked than the energy deficiency, and this results in decreased protein synthesis and edema. Children with marasmic–kwashiorkor have some edema and more body fat than those with marasmus.

Marasmus is the predominant form of PEM in most developing countries and can affect all age groups. Kwashiorkor is less common and is usually manifested as marasmic–kwashiorkor, especially in young children. It tends to occur where the staple foods are low in protein and excessively starchy (for example, yam [Dioscorea], cassava, sweetpotato, taro, plantain, and banana).

The assessment of PEM uses indices derived from measurements of body stature or length, weight, age, and presence or absence of edema that are compared with a reference population considered to have normal growth characteristics---usually the United States, although this has been questioned. The indices are underweight (low weight for age), stunting (low height for age), and wasting (low weight for height). National and regional level data on nutritional status have been compiled systematically for more than 20 y by the WHO Global Database on Child Growth and Malnutrition, which represents most of the children worldwide (de Onis and others 2004). Because the population in Asia is larger than elsewhere in the world, the number of undernourished is greatest in Asia, followed by Africa, although the proportion that are undernourished is greatest in Africa (de Onis and others 2004).

Views on the causes of PEM have evolved over time. By the 1960s, for example, it was known that the supply of protein in cereal-based diets was adequate (Hegsted and others 1946). The FAO nevertheless considered protein deficiency to be serious and widespread in developing countries. This was based on the observations that kwashiorkor, which was common among 1- to 4-y-old children in developing countries, (1) responded to therapy that included relatively high-protein supplements and (2) caused liver damage. Because liver cirrhosis was common among adults in Africa, the FAO thought this too indicated that African diets remained protein-deficient throughout life and the same might also be true in all developing countries. Milk and milk powder were expensive and in short supply. Consequently, much work was carried out to develop and test economical alternatives to milk powder based on locally available cereals and oilseed flours, fish protein concentrate, and single-cell protein from fermenting microorganisms. The work ended once it was realized that most children with kwashiorkor had been raised on diets that were as deficient in energy as they were in protein. The diets were also too bulky, which meant children could only eat a limited and inadequate amount (Whitehead 1973). Consensus was reached that the solution was to provide more high-energy foods and correct electrolyte deficiencies rather than concentrate just on protein (Waterlow 1961; McLaren 1974; Carpenter 1994), although it was recognized that diets based on staples such as cassava, bananas, and yams (Dioscorea) that are both very bulky and in low in protein present special challenges (Nicol 1971). In addition, initial treatment for severe PEM requires comparatively low levels of protein with a high biological value to ensure optimal utilization of protein for tissue maintenance and to prevent overloading the impaired metabolic system with superfluous amino acids and their nitrogenous metabolites. Following this initial treatment, high-protein diets such as milk-based formula can be used to stimulate growth and recovery (for example, Müller and Krawinkel 2005).

The importance of good nutrition starts at conception because fetal growth and development lay the foundation for health at all later ages. A fetus depends on the mother's ability to deliver nutrients, and this is greatly influenced by her nutritional state at the time she becomes pregnant as well as throughout pregnancy. Poor fetal growth and development have major consequences for the offspring, and these also have implications for health in the neonatal period, infancy, childhood, adolescence, and later life. Undernutrition in early childhood has serious consequences as these children are not only more prone to illness, but they also tend to have more severe illnesses than well-nourished children. Undernourished children are also at greater risk of dying. Undernutrition in school age children, much of which probably started in early childhood, adversely affects school attendance, performance, and learning. A stunted girl is more likely to become a stunted adolescent and later a stunted woman. Apart from direct effects on her health and productivity, undernutrition in an adult woman increases the chance that her children will be born with low birth weight, and the cycle begins again (United Nations Standing Committee on Nutrition 2004). Interventions to control undernutrition are therefore needed at different stages in the life cycle. These include improved child care and feeding practices for infants and young children; the provision of specific micronutrients through food fortification, biofortification, and pharmaceutical supplementation; improved access to health care; household

food security; good hygiene and sanitation; and universal female education.

The WHO global strategy for feeding infants and young children highlights the importance of good feeding practices during the transition period between exclusive breastfeeding and full weaning, when breastfed children also need complementary food. In addition to being safe and timely, the diet must be adequate—defined as providing sufficient energy, protein, and micronutrients to meet a growing child's nutritional needs (WHO 2002). Children need a variety of foods to ensure that nutrient needs are met. Diets that lack animal source foods (meat, poultry, fish or eggs, plus milk products) cannot meet the nutrient requirements for children ages 6 to 24 mo unless fortified foods or supplements are used. If milk and other animal source foods are not taken in adequate amounts, both grains and legumes should be consumed daily, preferably within the same meal, to ensure adequate protein quality (WHO 2004). In addition to the availability of these foods, their accessibility to the population depending on, for example, infrastructure and economic resources needs to be considered.

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Event-independent studies can include biochemical analysis (function, specificity and mode of action), studies on toxicity and the allergenic potential of the newly expressed proteins. In addition, dietary exposure modeling and bioavailability studies can be conducted with the newly expressed proteins and, in the case of enzymes, with the products produced by catalysis.

Event-dependent studies vary by country and can encompass the characterization of the quality of integration, such as copy number, the determination of the site of integration in the host genome, demonstration of the absence of gene disruption, completeness of integrated DNA sequences, and the presence of the marker gene at the same locus and absence of vector DNA sequences. Phenotypic and biochemical evidence for trait stability, including the monitoring of gene expression levels at key growth stages and Mendelian inheritance over several generations, are usually components of these studies. Field performance of typical agronomic traits, such as yield, and pest and disease resistance, is an additional issue. The assessment is also based on the comparison of the compositional analyses from materials harvested at different locations with the equivalent nontransgenic crop (and food) comparator. Event-dependent regulatory data are usually not gathered until the developer is satisfied with the technical performance of the product and a few events (usually one) have been chosen. Assessment of the potential risks of GM crops is on a case-by-case basis within a scientific framework. A GMO crop producing β-carotene must be viewed differently than a crop that produces an insecticide. Similarly, GR events expressing a phytoene synthase from maize, a widely consumed crop, might be viewed differently than one using the enzyme from a nonfood plant, such as daffodil.

From a scientific point of view it is often hard to understand why randomly mutagenized crop plants can enter into breeding lines easily, whereas GMOs, with considerably fewer genetic modifications, have a much more rigorous assessment. To have rational, science-based regulatory requirements it would be necessary to realize the benefits (not only potential risks) that GMO crops can provide (see lecture by Ingo Potrykus at http://www.syngentafoundation.com/golden_rice/index.htm). It is unhelpful to raise the regulatory requirements to the point where anything that appears technically feasible is being requested or is being offered to be applied (Kuiper and others

2001), with the only justification that the genetically modified organism involved is little understood. This strategy can raise costs to unaffordable levels. We do not understand bred varieties with their complex genomic changes much better at the molecular level, but we tend to consider the traditional way of producing new varieties safe in contrast to the novel way of producing GMOs even though this is not based on any rational evaluation. It is the final product and not the technology used to produce it that should be scrutinized.

References

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