SPECIAL GUEST EDITOR SECTION

Nutritional and Safety Assessments of Foods and Feeds Nutritionally Improved Through Biotechnology: Lysine Maize as a Case Study

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During the last decade, the area of biotech crops modified for agronomic input traits (e.g., herbicide tolerance and insect protection) has increased to 90 million ha/year, grown by over 8 million farmers in a total of 17 countries. As adoption of these improved agronomic trait biotech crops has grown, so has interest in biotech crops that have improved nutritional characteristics for use as feed and food. A previous publication by the International Life Sciences Institute (ILSI) reported on the principles and concepts proposed for the nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology. In this paper, the guidelines and principles recommended in the earlier publication are discussed relative to a specific case study, Lysine maize. Lysine maize is a feed ingredient with enhanced nutritional characteristics for poultry and swine and provides an alternative to the need for addition of supplemental lysine to some diets for these animals. The 2004 Task Force of the ILSI has also applied the concepts from that report to 4 other case studies: sweet potato enriched in provitamin A (2 examples, one using biotechnology and one using conventional breeding); *Golden Rice* 2; double-embryo maize; and ASP-1 enhanced protein sweet potato.

In 2001, a Task Force of international scientific experts was convened by the International Food Biotechnology Committee (IFBiC) of the International Life Sciences Institute (ILSI) to address the topic of the safety and nutritional assessments of foods and feeds that are nutritionally improved through modern biotechnology. In 2004, the work of the Task Force culminated in the publication of a report that included a series of recommendations for the nutritional and safety assessments of such foods and feeds (1). This document has gained global recognition from organizations such as the European Food Safety Agency (2) and has been cited by Japan and Australia in 2005 in their comments to Codex Alimentarius.

The same Task Force of scientific experts has now applied the principles recommended for the safety and nutritional assessments of nutritionally enhanced crops set forth in the 2004 ILSI publication to a set of 5 case studies of nutritionally enhanced food and feed crops currently in development through either conventional breeding or the application of modern biotechnology. This Task Force will be publishing its assessment of these 5 case studies in 2007. The case studies have been used to explore whether the concepts and recommendations in the 2004 ILSI publication provide a strong and robust paradigm for the safety assessment of "real world" examples of nutritionally improved crops. A set of recommendations that were observed to be consistent across the 5 case studies has been developed by the Task Force. These recommendations confirm the soundness of the concepts set forth in the previous 2004 ILSI publication. In the present paper, one of those improved nutrition case studies, Lysine maize, will be assessed relative to the set of recommendations that are consistent across the safety and nutritional assessment of the 5 case studies.

Background on the Importance of Nutritionally Improved Foods and Feeds

During the last 2 decades, the public and private sectors have made substantial research progress internationally towards 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 improved nutrition come closer to being available to the consumer, we must ensure that scientifically sound and efficient processes are used to assess the safety and nutritional quality of these crops. Such a process will facilitate deployment of these crops to those world areas that have large numbers of people who need them.

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 form of

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hunger and malnutrition. 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 a reduction in 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 have 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.

Plant scientists have worked diligently to improve the nutrient content of staple crops consumed in developing countries. In addition to using the natural variation present in crop germplasm, the tools of modern biotechnology 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.

Background on the Lysine Maize Case Study

Nutritional limitations to livestock production are numerous and varied in both developed and developing counties. Animal production is often restricted because feed resources are deficient in one or more nutrients (e.g., maize grain is typically low in lysine content compared to other crops); have limited nutrient bioavailability (e.g., the use of crop residues as the primary forage source in developing countries); or are constrained by the presence of antinutritional factors or toxins (e.g., phytate and mycotoxins). Several crops have been developed and are currently in trials with biofortification aimed at improving nutritional characteristics in which the concentration of a specific nutrient, such as an essential amino acid, is increased. For nonruminant livestock production systems, maize grain is often the preferred energy source; however, it is low in some of the essential amino acids, including lysine. Thus, in developed countries, some poultry and swine diets based on maize and soybean meal include supplemental crystalline L-lysine for optimal animal growth and production (3, 4). Supplemental L-lysine is commercially available and is typically produced via fermentation of *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* (5). Lysine maize, LY038, was developed through the use of recombinant deoxyribonucleic acid (DNA) techniques to integrate into the maize genome the *cordapA* gene that results in the production of maize grain with higher lysine content. This grain with enhanced lysine concentration has improved nutritional value, simplifying its use as a feed ingredient in diets for broiler chickens, turkeys, and pigs by reducing or eliminating the need to supplement with crystalline lysine.

To produce Lysine maize, a linear piece of DNA from a plasmid vector containing the *cordapA* and *nptII* coding sequences was introduced into maize. 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 to be propagated. The *nptII* gene cassette was subsequently removed from Lysine maize using cre/lox technology (6).

The *cordapA* gene is from *C. glutamicum*, a common soil microorganism that has been used for decades in the industrial production of L-lysine (7). Dihydrodipicolinate synthase (DHDPS) is the first and major rate-limiting enzyme for lysine biosynthesis in plants and bacteria (8) and is regulated by lysine feedback inhibition (9). However, the DHDPS from *C. glutamicum* (cDHDPS) is comparatively insensitive to lysine inhibition (10). Expression of the *cordapA* gene in LY038 plants is under the control of the *Zea mays globulin* 1 (Glb1) promoter, resulting in expression of the *cordapA* gene in the grain that results in increased free lysine and natural metabolites of lysine in the embryo portion of the kernel (11).

Southern blot analyses showed that LY038 contains 1 intact copy of the *cordapA* gene cassette inserted at a single site in the maize genome. 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 lacks detectable backbone sequence from the transformation plasmids. The presence of the *cordapA* gene cassette and absence of both *cre* and *nptII* gene cassettes in LY038 was 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.

The Lysine Maize Case Study Relative to the Task Force Recommendations

The IFBiC Task Force made a set of recommendations that were observed to be consistent across 5 case studies of improved nutrition crops assessed by the Task Force. These recommendations confirmed the soundness of the concepts set forth in the previous 2004 ILSI publication. Presented below is each recommendation relative to the Lysine maize case study.

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.

One overarching conclusion from the work of the Task Force that is evident in the Lysine maize case study is that the comparative safety assessment process is applicable. It is well recognized that absolute safety is not an achievable goal in any human endeavor, and this is particularly 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 experts who determine its safety based on established scientific procedures. Starting in the 1990s, the standard applied to novel, especially biotech, food and feed crops has been that they should be as safe as an appropriate counterpart that has 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 that has a history of safe use. This assessment process has been endorsed by many publications and organizations, including the 2004 ILSI publication (1, 2, 12–19). The comparative safety assessment process has sometimes been called the "substantial equivalence principle."

Key to the comparative safety assessment process is the recognition that the comparative analysis of composition and plant phenotypic properties is the starting point, not the conclusion, of the assessment. 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 the traditional crop with a history of safe consumption. The identified differences are subjected to further scientific assessment to clarify whether any safety issues or concerns exist. 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.

A fundamental aspect of the comparative safety assessment process is the use of a comparator with a history of safe consumption. To date, the comparator has been a traditional crop developed through conventional breeding, since a long history of safe consumption exists in such instances. Therefore, it is interesting to note that recent studies have characterized the genetic changes that occurred historically during plant evolution, crop domestication, and the many forms of "conventional breeding" (20–27). It has been shown that 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 application of modern biotechnology. For example, "the occurrence of unintended effects is not unique for the application of recDNA techniques, but also occurs frequently in conventional breeding" (28) and "in fact, conventional breeding programs generally evaluate populations with much wider ranges of phenotypic variation than is observed in transgenic programs..." (29). Thus, the history of safe consumption of domesticated crops has been possible while those plant genomes have undergone large changes.

In addition, it has become clear that major domesticated crops have a wide genetic diversity in the various global environments in which they are grown, and that such diversity is possible because breeding often coselects for hypermutable, genetically fluid cultivars. Extensive variation in DNA content is normal within a species, in which DNA movement and rearrangements are common, natural phenomena (30–32). Individual plants within a species can obtain, through natural or directed selection, differing numbers of genes and/or number of whole chromosomes.

Variations due to breeding and to the application of modern biotechnology have frequently been studied by scientific experts sponsored by organizations such as the Food and Agriculture Organization of the United Nations (FAO), the European Commission, and the (U.S.) National Academy of Sciences. In each case, the conclusions were that modern biotechnology is no more likely than conventional breeding to produce unintended effects (2, 13, 32). Indeed, many expert reviews have concluded that the greater precision and more defined nature of the changes introduced into crops via modern biotechnology may be safer than changes produced by conventional plant breeding that has a history of being safe in and of itself (29–31).

With Lysine maize, as with all crops derived from modern biotechnology, to be commercially successful, they undergo extensive breeding with elite lines such that >99% of the germplasm of commercialized hybrids will be derived from elite lines that have not experienced genetic transformation. This breeding significantly reduces the opportunity for transformation-induced, random genome changes from being in the final product. As described below in more detail, extensive analysis of both the composition and the phenotypic properties of Lysine maize showed that no changes were detectable, outside of the intended increase in grain lysine content and the associated increase in natural metabolites of lysine.

The comparative safety assessment of Lysine maize detected only the anticipated changes associated with the increase in lysine; therefore, these changes must be subjected to additional safety assessment. Lysine is an essential amino acid and is Generally Recognized As Safe when added to animal diets at nutritional levels (33) and may be safely used as a human food additive when used as a nutrient (34). Furthermore, excessive consumption of lysine by humans, pigs, and rats over prolonged periods is well tolerated (35). In plants and animals, lysine is primarily catabolized via the saccharopine pathway by 2 linked enzymes, lysine-ketoglutarate reductase and saccharopine dehydrogenase (36).

Because ingested lysine is largely degraded through the saccharopine pathway in humans, animals, and plants, tissues are 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 saccharopine dehydrogenase (37). 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 production. 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 broiler chickens and rats provide supporting data regarding the safety of Lysine maize grain for humans, because maize grain exposure levels were orders of magnitudes higher for the animals than potential human consumption levels for maize grain-based products. Therefore, there is reasonable certainty that the increase in Lysine maize of free lysine as well as the associated increase in the natural metabolites of lysine, saccharopine, and α-aminoadipic acid are not expected to be harmful to either animal or human health.

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.

Today, maize ranks third after wheat and rice as one of the world's 3 leading food grains; it is grown on 140 million ha/year in 100 countries, and 700 million metric tons of grain were produced in 2004. The major producers of maize are the United States, the People's Republic of China, Brazil, Mexico, France, and India, accounting for 75% of world production (13). However, unlike wheat and rice, the majority of maize grain produced in the northern hemisphere is fed to livestock, whereas in the tropics and the southern hemisphere, maize is a major staple food for humans.

While maize grain is often the preferred dietary energy source for both ruminant and nonruminant livestock production systems, it is recognized that in some diets for pigs and poultry, supplementation with crystalline L-lysine can optimize animal performance.

Lysine maize will be a value-added specialty crop for use as an animal feed ingredient. 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 ≈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 (≈1500 mg/kg). Usage of Lysine maize in lieu of conventional maize can simplify diet preparation by eliminating the need for crystalline lysine in many diets. Usage of Lysine maize is not expected to result in an increase in total dietary lysine, since diet formulation can follow current industry best practices. 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.

Production and processing of feed from Lysine maize are not expected to differ from those of conventional maize, although usage of Lysine maize in lieu of conventional maize would simplify feed preparation by eliminating the need for supplemental crystalline L-lysine in many animal diets. 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 is 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. In addition, in the unlikely case that adventitious amounts of Lysine maize grain enter the food chain, free lysine and related natural metabolites will most likely fractionate in the animal feed components and not those used for human foods.

Recommendation 3.—The safety of any protein(s) newly introduced into a crop needs to be assessed. It is noted that recommendations for the safety assessment of transgenic proteins follow a tiered approach that are currently being finalized for publication by an ILSI IFBiC protein safety Task Force.

The development of Lysine maize, like several of the case studies assessed by the Task Force, involves introduction of 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 recommendations for a framework for the safety assessment of proteins. The report from this protein safety Task Force, which is expected to be published by early 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. Both Codex Alimentarius (38) and the European Food Safety Agency (2) 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 it has 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.

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, while the endosperm is the predominant fraction consumed by humans. Nonetheless, the safety of cDHDPS was assessed for both food and feed applications.

C. glutamicum is a common soil bacterium to which animals and humans are regularly exposed without adverse consequences. All *C. glutamicum* cultures available from the American Type Culture Collection (Manassas, VA) are

classified at Biosafety Level-1, the safest of all cultures as defined by the U.S. Department of Health and Human Services (39). In addition, DHDPS proteins structurally and functionally related to cDHDPS in Lysine maize are present in plants and microbes that make lysine, many of which are consumed as feed and/or food, such as maize.

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

In vitro digestibility and acute mouse toxicity studies with cDHDPS utilized protein produced and purified from *Escherichia coli*. Before initiation of these studies, however, the equivalence of cDHDPS produced in *E. coli* to the maize-produced cDHDPS expressed in Lysine maize was determined 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, and N-terminal sequence analysis. From these experiments, cDHDPS purified from *E. coli* was shown to be physicochemically and functionally equivalent to cDHDPS produced in Lysine maize.

Previous studies have assessed the susceptibility of proteins expressed in genetically modified plants to proteolytic digestion in vitro (40) following a standard protocol (41). Recently, ILSI standardized the pepsin digestibility assay protocol through a multilaboratory evaluation (42). The in vitro digestibility of *E. coli*-produced cDHDPS 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 protein digestibility of cDHDPS 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 cDHDPS in SGF, it can be inferred that >96% of cDHDPS was observed to be digested in SGF within 30 s. Western blot analysis confirmed that >98% of cDHDPS was digested below the LOD of this immunoassay within 30 s of incubation in SGF. No stable peptide fragments of cDHDPS 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 cDHDPS has low allergenic and toxic potential.

An acute high-dose oral toxicity study was considered appropriate to assess the safety of *E. coli*-produced cDHDPS. In this study, 2 groups of 10 animals/sex were given a single gavage dose of 800 mg/kg body weight cDHDPS. A 14-day period followed the administration of cDHDPS, during which the mice were observed daily and weighed weekly. There was no mortality and no reports of adverse clinical reactions. All cDHDPS-dosed mice gained weight and consumed food during the 14-day postdosing period comparable to control mice. A gross necroscopy examination was conducted on all animals at study termination. At necropsy, 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 cDHDPS was determined to be >800 mg/kg body weight, the highest dose tested.

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. 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 for broiler chickens and pigs) and humans (>45 000 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.

Recommendation 4.—Compositional analysis of crops with known toxicants 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.

In biotech crops with improved nutritional characteristics, metabolic pathways are often modified to achieve the desired nutritional improvement, and a full understanding of the changes that have occurred is important for both the safety and nutritional evaluation of the biotech crop. The metabolic pathways altered in the development of Lysine maize are well characterized and are shown in Figure 1 for lysine anabolism and in Figure 2 for lysine catabolism in maize.

Because the lysine metabolic pathways are well understood, there was no need to employ untargeted compositional analysis of Lysine maize. Within one chapter of the 2004 ILSI publication (1), 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. However, even if the lysine pathways in maize were not well understood, untargeted compositional analysis would not have been informative, because efforts

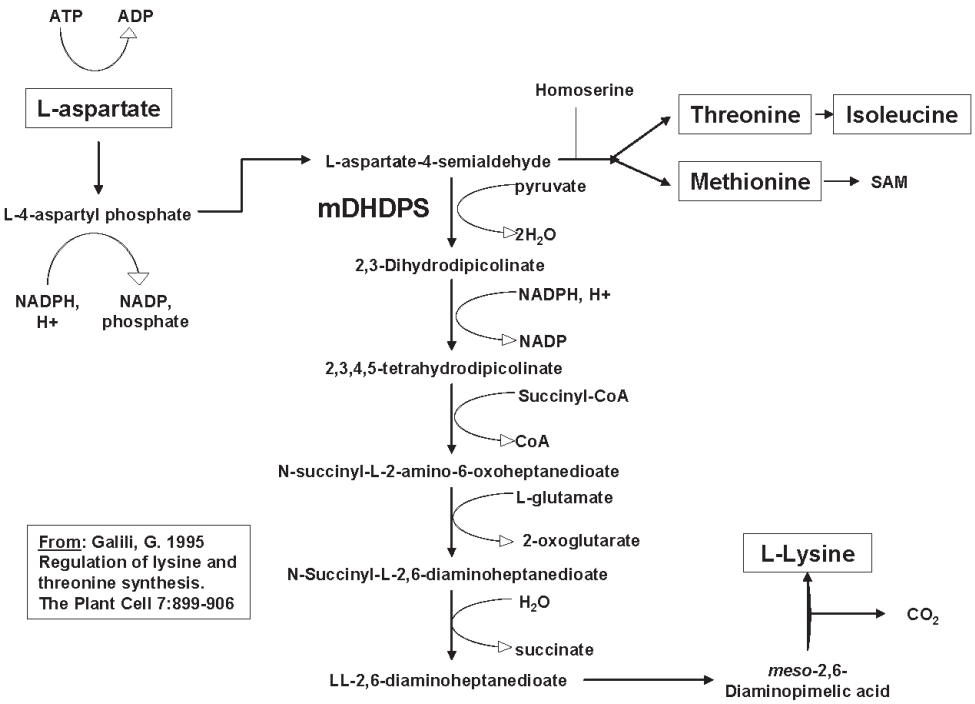

Figure 1. Lysine anabolism in maize.

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 http://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 against which profiles of metabolites and proteins in novel foods may be compared, if deemed necessary.

Compositional analysis is considered as the cornerstone for the safety and nutritional evaluation of biotech crops. In the current case study, extensive compositional analyses of forage (whole plant at early dent stage) and grain were conducted on samples from replicated, multisite field trials conducted in both Argentina (2001–2002) 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 and phosphorus), as well as carbohydrates by calculation. Compositional analyses of Lysine maize grain samples also included proximates; ADF; NDF; total dietary fiber; total amino acids; fatty acids (C8–C22); vitamins (thiamin, riboflavin, B_6, 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 (furfural, ferulic acid, and *p*-coumaric acid) according to the Organization for Economic Cooperation and Development (OECD) consensus document (43); and additional lysine-related metabolites (free lysine, 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 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 the grain and forage of conventional maize, except for the intended increase in grain lysine content and an associated increase in 2 of the natural lysine-related catabolites, saccharopine and α-aminoadipic acid. None of the other lysine-related metabolites measured was significantly different from the near-isogenic control and/or outside the natural range in maize grain. Together, this composition data support the conclusion that there were no unintended changes in Lysine maize due to the genetic modification, outside of the

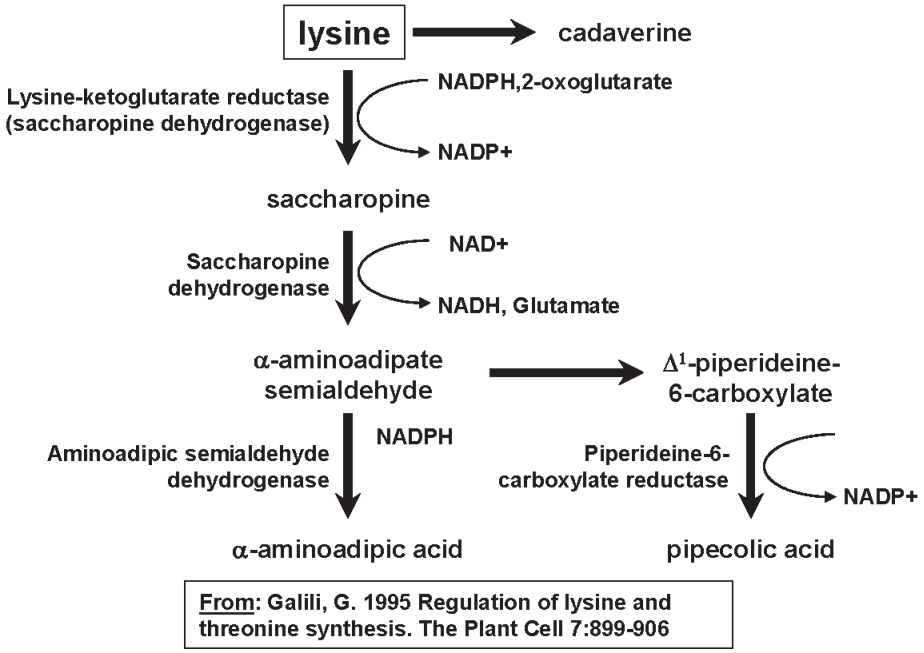

Figure 2. Lysine catabolism in maize.

intended improvement in total lysine content of the grain, and the associated expected increase in natural lysine-related metabolites.

Recommendation 5.—The 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 are identified.

To assess whether unintentional changes because of genetic modification resulted in an altered phenotype of Lysine maize, extensive measurements of phenotypic and agronomic properties of Lysine maize were compared to those of conventional maize plants grown at the same time in field trials using a randomized complete block design at multiple locations in Argentina and the United States. Phenotypic characteristics (e.g., 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 between 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 from this data (e.g., no differences detected in the mode or rate of reproduction, maize grain dissemination, or survivability), plus other data not reviewed in this document (e.g., lack of observed impact on insect and other wildlife), that Lysine maize is as safe for the environment as conventional maize. It can also be concluded that the agronomic and phenotypic characteristics of Lysine maize are equivalent to those of conventional maize.

Recommendation 6.—Studies in laboratory animals may serve a useful role in confirming observations from other components of the safety assessment, thereby providing a sense of added safety assurance. Any nutritional animal feeding studies, if conducted, need to be performed with a suitable species that, for animal feed products, should include the target species, and should follow the guidances formulated by the ILSI Task Force on "Best Practices for the Conduct of Animal Studies to Evaluate Genetically Modified Crops" (44). However, studies in laboratory animals and targeted livestock generally lack adequate sensitivity to reveal unintended minor changes that have gone undetected by targeted analysis.

The safety of Lysine maize grain has been further assessed by a 90-day feeding study in rats. 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 days.

Broiler chicken and swine diets based on maize and soybean meal may require the addition of supplemental lysine for optimal animal performance (3, 4). Supplemental lysine is usually in the form of lysine monohydrochloride or lysine sulfate (45) produced via fermentation by *C. glutamicum* or *B. lactofermentum* (5). Both of these lysine sources are highly bioavailable (46–48), 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 (49). 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 (50–53).

A study with fast-growing broiler chickens was carried out to compare the performance (growth rate, feed efficiency, and carcass characteristics) of Lysine maize and the bioefficacy and bioavailability of the lysine in Lysine maize when compared with diets containing either a control maize grain with comparable genetics to Lysine maize or each of 4 reference maize varieties. Each of the control and reference maize diets was formulated with and without supplemental crystalline L-lysine so that the diets with the supplemental lysine had a similar dietary lysine concentration compared 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 the health and performance of broiler chickens.

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 broiler chickens 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.

The last 2 recommendations (7 and 8) are not relevant to Lysine maize, since this product is intended to be used for animal feed. Recommendation 7 focusing on premarket nutritional impact studies in humans of the improved nutrition crop might be appropriate, depending upon the intended nutritional change, but should not be triggered solely based on how the crop was developed. Such an assessment could study the biological or biochemical effectiveness of the intended trait and/or to determine if the introduction of the improved nutrition crop will adversely change nutrient intake. Recommendation 8 focuses on the scientific assessment of the possible consequences of the adoption of improved nutrition crops and should balance not only assessing the potential risks, but also the opportunity for benefits to alleviate undernutrition for a potentially large number of people.

Conclusions

The crops being developed to improve human or animal nutrition hold great promise in helping to address global nutrition needs. The data and information in the Lysine maize case study provides an example of a biotech crop in which a specific nutrient has been increased and 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 biotech crops is also appropriate and recommended for the safety and nutritional assessment of nutritionally enhanced crops derived through modern biotechnology. Additional studies may be needed for specific cases to assess potential safety or nutritional consequences resulting from changed levels of the improved nutritional factor(s). Such studies, for example, might need to focus on the level of the components in the biosynthetic and degradative pathways for the increased nutrient. For both conventional and biotech crops, the breeding and development process (e.g., selecting a single commercial product from large numbers of crosses between conventional lines or from hundreds to thousands of initial transformation events for biotech crops) eliminates the vast majority of conventionally bred varieties and biotech events that contain unintended changes. 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.

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, because this provides evidence that the new crop is as safe as the conventional counterpart with a history of safe consumption. The identified differences, then, become the focus of additional scientific assessment.

For the present case of Lysine maize, the available data show that it is as safe as conventional maize while being nutritionally enhanced for animal feed diets.

Specifically, for Lysine maize, 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 a diet containing conventional maize grain and 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 Lysine maize to conventional maize hybrids. Results of the comparative safety assessment studies demonstrated that Lysine maize grain is substantially equivalent to conventional maize, with the exception of the intended increase in lysine and the corresponding increase in 2 natural lysine catabolites. (*3*) Safety assessment of the newly introduced protein, cDHDPS, with respect to allergenicity and toxicity showed no major concerns.

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