Agricultural Biotechnology

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Agricultural Biotechnology

Challenges and Prospects

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

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Preface

The majority of agricultural scientists, including myself, anticipate great benefits from biotechnology in the coming decades to help meet our future needs for food and fiber.

Norman E. Borlaug (*1*)
Nobel Prize Laureate for Peace, 1970

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Agricultural biotechnology has provided powerful tools to enhance the modification of plants to the potential benefit of society. Since the introduction of the first biotechnology product, the Flav Savr™ tomato, in 1994, followed by the introduction of Roundup Ready® soybeans and Bollgard® cotton in 1995–1996, the number of acres planted with biotechnology-derived crops has steadily increased. In 2002, the global area of biotech crops was 145 million acres, representing an increase of 12% or 15 million acres more than 2001 (*2*).

However, the introduction and rapid adoption of this technology has not occurred without controversy. Questions have been raised about the food and feed safety of these new products and their effect on the environment. These issues have highlighted the need for communication of the experimental evidence and scientific evaluation for the assessment of the benefits and any risks to society (*3*). The intent of this book is to provide up-to-date information on the critical issues facing agricultural biotechnology as the 21st century unfolds. It is our hope that these presentations of research by key scientists involved in agricultural biotechnology will place information in the hands of both technical and non-technical citizens so that they may make informed decisions that will shape the future of this technology.

The contents of this book were based upon two agricultural biotechnology symposia, one sponsored by the American Chemical Society (ACS) Division of Agricultural and Food Chemistry and the other sponsored by the ACS Division of Agrochemicals. Both symposia were held during the ACS National Meeting in Chicago, Illinois, in August of 2001. In addition to the chapters of the book, an introduction (Chapter 1) was added to provide background information for the reader

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on topic areas not directly addressed in the book's chapters. Chapter 1 contains brief descriptions of the development of agricultural biotechnology, the U.S. regulatory process for biotechnology products, the importance of this technology for the developing world, and finally a look toward future prospects and the products that could help meet the needs of agricultural producers and consumers during the next five to ten years. The coverage of the topics in Chapter 1 was not intended to be comprehensive, so references are provided to assist readers who are interested in more detail.

The chapters of this book are divided into three sections: Benefits: Present and Future; Analytical Methodologies; and Food, Feed, and Environmental Safety Assessment. Chapter 2, in The Benefits: Present and Future section, by Nicholas Kalaitzandonakes discusses the future of agricultural biotechnology from the perspective of national competitiveness. Kalaitzandonakes points out that apparent dissonance between technology providers and users has led to conflicting views on the future of this technology. He takes a look at technical innovation, institutions, and markets to assess the future prospects for agricultural biotechnology. Chapter 3 by Janet Carpenter and colleagues presents a discussion of the benefits of crops containing the *Bacillus thuringiensis* (*Bt*) insecticidal protein when compared to the use of conventional chemical insecticides. These authors conclude that corn growers have increased yields whereas cotton growers have significantly reduced insecticide use when using these crops. In Chapter 4 of this section, Ann Blechl presents a review of the research on improving the food and non-food uses of cereal crops using the tools of agricultural biotechnology. Because a majority of the world population derives a very significant portion of its calories and nutrients from the cereal crops, including rice, wheat, maize, and barley, studies of this type could have a significant influence on global nutritional health.

Chapter 5 by James Stave and coauthors is the first of two chapters in the Analytical Methodologies section. Proteins are responsible for the unique biological traits in the current herbicide tolerant and insect-protected products of agricultural biotechnology. Stave presents a discussion of the essential role of immunoassays for the detection of these proteins in both grain commodities as well as processed fractions, and the potential role of these assays for compliance with food labeling requirements. The use of polymerase chain reaction (PCR) for the detection of DNA from plant-derived materials is the subject of Chapter 6 written by Matthias Klaften and coauthors. These authors discuss the

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challenges of using PCR assays to detect the presence of maize rubisco gene in the tissues of animals fed maize in the diet and the safeguards necessary to prevent false positives when using these extremely sensitive assays.

The final section of the book, Chapters 7–12, deals with the broad subject of Food, Feed, and Environmental Safety of biotechnology products. Bruce Hammond in Chapter 7 discusses the safety and benefits of insect-protected crops that contain *Bt* Cry proteins. The Cry proteins are a family of insecticidal materials that specifically bind to receptors in the intestinal tract of susceptible insects. Because these receptors are not present in vertebrates such as humans, the crops containing *Bt* are toxic to the insects but they are safe for vertebrate species. In the next chapter, Chapter 8, Mark Sears presents the results of a collaborative research project to determine the effect of *Bt* pollen on Monarch butterflies under field conditions. The conclusions from this work are significant because the initial observations from laboratory studies created a concern about the environmental effects of *Bt* crops on nontarget insects. Chapter 9 contains a discussion by Kevin Steffey and coauthors of the comparison of the effect of *Bt* and non-*Bt* corn fields on the natural enemies of the European corn borer.

In Chapter 10 Gary Bannon and colleagues describe the studies that are typically conducted to determine the potential for an allergic response to the protein introduced in food from an agricultural biotechnology product. The safety assessment is a tiered testing strategy that uses available scientific data on known allergens and the allergic response. Chapter 11 describes how the analysis of the nutritional composition of a biotechnology crop is compared to that of its conventional counterpart as part of the evaluation of substantial equivalence. This process, described by William Ridley and coauthors, also emphasizes that it is important to account for the natural variability in composition that occurs as a result of the genetic background and environment of the crop plants when making these comparisons. The final chapter of the book, Chapter 12, is by J. Clark and I. Ipharraguerre in which they review the extensive data available on the animal nutrition of biotechnology products. Studies have been conducted with a wide array of livestock species fed diets containing material from genetically modified corn, soybeans, canola, cottonseed, and sugar beets. The results indicate that the animals develop in a comparable manner and the food products derived from these animals are safe for human consumption.

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Chapter 1

Introduction to Agricultural Biotechnology: Challenges and Prospects

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The emergence of agricultural biotechnology was dependent upon advances in our understanding of the basic biochemical, genetic and physiological processes of biological systems and has led to the development of products that have provided farmers with new tools to increase productivity and decrease the environmental impact of agricultural practices. This chapter contains an overview of the history of agricultural biotechnology, its regulation in the United States and its importance to developing countries. The prospects for the future development of new products with enhanced pest and disease resistance and nutritional properties are also presented.

A Brief History of Agricultural Biotechnology

Jared Diamond in his wonderfully interesting book, " Guns, Germs, and Steel: The Fates of Human Societies" (1) describes the importance of the domestication of wild stocks of plants that became the crops of wheat and barley in the valley of the Tigris and Euphrates rivers that is now called the Fertile

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Crescent. Domestication of plants and large mammals enabled peoples to settle down: surpluses of food could be produced and stored and so the population grew. As early as 10,000 years ago agriculture was born and the development in our understanding of the basic biology of plants and animals has paralleled the development of human society.

This brief description of the author's view of the history of agricultural biotechnology will, by necessity, be subjective. There are many key events that were not included or that may have been inadvertently excluded. More detailed reviews are available (2, 3) and therefore this section will attempt to select only a few of the many scientific milestones that have led to the discipline known as "agricultural biotechnology". The emphasis will be on those breakthroughs and issues that have effected the development and acceptance of agronomic food and feed crops containing specific traits derived through biotechnology.

Gregor Mendel (1822-1884) was the first person to understand that the inheritance in peas (*Pisum sativum*) of specific traits such as flower color, seed shape and stem length occurred in completely predictable and quantitative ways (4). In a very real sense, Mendel invented the science of genetics and his observations provided the basis for the future development of crop breeding and hybrid crops.

In the early 1930's hybrid corn was commercialized based on the work of Henry Wallace and others in the 1920's. Also in 1931, Linus Pauling published the first in a series of papers (5) describing the nature of the chemical bond. These studies were included in Pauling's classic book, "The Nature of the Chemical Bond and the Structure of Molecules and Crystals" (6) that laid the foundation for the quantitative estimate of bond lengths and bond angles that are crucial to the definition of chemical structure.

The debate over the nature of the hereditary material was resolved by the definitive experiment performed in 1944 by Oswald Theodore Avery, Colin MacLeod and Maclyn McCarty (7) who determined that deoxyribonucleic acid (DNA) must be the hereditary substance responsible for the tranformation from avirulent to virulent in pneumococcus bacteria. The work of many researchers including Rosalind Franklin, Maurice Wilkins, Jerry Donohue and Erwin Chargaff contributed to the proposal of a chemical structure for DNA in 1953 by James D. Watson and Francis Crick (8). This structure was consistent with the experimental data, was esthetically beautiful and also provided an explanation for how DNA was replicated and how parents pass hereditary information to their progeny. In 1961, Marshall Nirenberg and colleagues (9, 10) proposed a language for the genetic code that was composed of "three letter words" written

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in the structure of ribonucleic acid (RNA) and then translated into the amino acid sequence of proteins.

Plasmids are small, circular molecules of DNA that occur in bacteria and yeast, and are replicated autonomously. Stanley Cohen and colleagues in 1973 described the formation of new plasmid DNA species by *in vitro* joining of fragments from other plasmids (11). These discoveries were followed by experiments demonstrating that the Ti plasmid of *Agrobacterium tumefaciens* , the causative agent in crown gall disease, could be modified by removing the tumor inducing genes and incorporating desired gene sequences which were then carried into plant cells as reported by Mary-Dell Chilton and her colleagues in 1977 (12).

The ability to regenerate plants using a leaf disk transformation method was accompanied by the demonstration in 1984 of the inheritance of specific traits in plants derived from transformed cells by Horsch and colleagues (13, 14, 15). The ability to amplify DNA using the polymerase chain reaction permitted researchers in 1985 to establish the presence of specific genetic traits in both plant and animal tissues (16). Another technical breakthrough occurred shortly thereafter with the demonstration in 1987 that microprojectiles coated with DNA could be used to transform living cells (17).

Field testing of transformed plants and safety product testing began with intensity during the late 1980's and this set the stage for the introduction in 1994 of the first food product derived from agricultural biotechnology, the Flavr Savr™ tomato (18). In the Flavr Savr™ tomato, the process of softening was slowed by copying the gene responsible for the softening process, reversing its orientation and then reintroducing the resulting "anti-sense" gene to switch off expression. The following two years, 1995-1996, witnessed the commercialization in the United States of two additional crops, Roundup Ready® soybeans, designed to be tolerant to the broad spectrum herbicide Roundup® and YieldGard® corn, which was protected against the European corn borer by the presence of a protein from the soil microbe, *Bacillus thuringiensis* (Bt) (19).

A report in 1999 by John Losey and colleagues suggested that pollen from N4640 Bt corn containing the Bt gene was toxic to monarch butterfly larvae (*Danaus plexippus*) when administered at high levels in the laboratory (20). Subsequent studies by a consortium of academic and governmental scientists demonstrated that the impact of Bt corn pollen on monarch butterfly populations under natural field conditions was negligible (21). The following year in 2000, the Environmental Protection Agency issued the results of a Scientific Advisory Panel analysis of the insect-protected product, Starlink® (22). There was no

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adverse effect demonstrated from the presence of StarLink corn in food products but because it had been approved only for animal feed, the product was voluntarily removed from commercialization and discontinued after being detected in the human food supply in the United States.

One aspect of the potential for agricultural biotechnology to enhance the nutritional value of food was demonstrated in 2000 by the development of "golden rice" by Potrykus and colleagues (23). The rice was named "golden" due to the color resulting from increased levels of β-carotene, the precursor of vitamin A. Through the efforts of Florence Wambugu and her colleagues, a sweet potato was engineered using the tools of agricultural biotechnology to be resistant to the feathery mottle virus. The sweet potato is a rich source of calories and vitamins for Sub-Shaharan Africa and the first field trials in Kenya of the virus resistant sweet potato were initiated in 2000 (24).

Regulatory Oversight of Agricultural Biotechnology in the United States

The development of new technology, particularly technology that affects food and animal feed, requires careful consideration of the results to make sure there are no safety concerns. Since the commercialization of the first products of agricultural biotechnology was in the United States (U.S.), the issues of safety to the food and feed supply and the environment were initially addressed by the U.S. regulatory agencies. The global importance of agricultural commodities such as soybeans, corn and processed fractions derived from these commodities has highlighted the interconnected and interdependent nature of food worldwide. Regulatory systems are currently in place or are in the process of development in Canada, the European Union, Japan and other Asian countries, Australia, Argentina, Brazil and parts of Eastern Europe. Such regulatory programs are essential to the worldwide acceptance of agricultural biotechnology however a detailed discussion of these processes is beyond the scope of this chapter.

As agricultural biotechnology was being developed, it was generally agreed by scientists, regulators and policymakers in the U.S. that plants developed with these new tools should be carefully evaluated before being released into widespread use. There have been several recent reviews of the U. S. regulatory systems for agricultural biotechnology (25, 26, 27). Three lead agencies, the United States Department of Agriculture (USDA), the Food and Drug

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Administration (FDA), and the Environmental Protection Agency (EPA), are responsible for implementing a "Coordinated Framework for Regulation of Biotechnology" that was developed under the auspices of the White House Office of Science and Technology and published on June 26, 1986 (28). The document also recognized a continuing role for the National Institutes of Health (NIH) for guidance on laboratory and greenhouse research (29). Under this framework, all biotechnology products are subject to premarket safety assessment by at least one federal agency in accordance with existing, product-based statutes. In general terms, plants remain subject to the USDA's jurisdiction, pesticidal substances in plants are regulated by the EPA, and food and feed are regulated by the FDA. Depending on the properties of the product, several agencies may be involved in the safety assessment.

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Nine steps have been identified in the U.S. governmental safety evaluation of the products of agricultural biotechnology as indicated below (25):

1. Biosafety Committee – National Institutes of Health Biosafety Guidelines
2. USDA greenhouse standards and inspections
3. USDA field trail authorization
4. USDA authorization of transport for field trials
5. USDA determination of non-regulated status
6. EPA experimental use permit approval
7. EPA determination of food tolerance or tolerance exemption
8. EPA product registration
9. FDA review process (voluntary premarket consultation)

Many of the steps listed above provide the opportunity for public input. Between 1987 and 2000, approximately 7,000 small-scale field tests were conducted under the USDA's Animal and Plant Health Inspection Service (APHIS) regulations. By early 2001, forty-nine plant products approved by the USDA had also completed the voluntary FDA premarket review process. Nineteen of these 49 biotechnology-derived plants were reviewed and registered by the EPA since they were either insect- or virus-resistant products hence triggering the "biopesticide" oversight authority of that agency (25).

The responsibilities of the individual U.S. regulatory agencies for agricultural biotechnology products are derived from existing statutes. APHIS of the USDA is responsible for assessing the environmental safety of biotechnology-derived crops under the authority of the Plant Protection Act (30). Crop developers must file a notification for permission to import, grow, or move across state lines any plant biotechnology product. The primary focus of the APHIS review is to determine whether or not a plant produced through

biotechnology has the potential to become a weed, create plant pests through outcrossing, or otherwise adversely affect environmental habitats.

The EPA regulates pesticides produced in biotechnology-derived plants by authority of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for "plant-incorporated protectants" (31). Under FIFRA, a pesticide must not cause unreasonable adverse effects on the environment, which by definition includes both ecological as well as human health effects. As a result, the EPA requires detailed information on the expression, specificity, toxicology, impacts on non-target organism, wildlife and endangered species, and environmental fate of new proteins in agricultural biotechnology products.

The primary authority for the safety of the food supply has been granted to the FDA by the Federal Food, Drug and Cosmetic Act (FFDCA). The legislative history of the FFDCA has reflected the realization that it is impossible to achieve absolute safety in the food system and therefore the FDA has defined the standard for food safety as "a reasonable certainty of no harm". Because each product is unique and poses different issues, there is no standard battery of tests specifically required for all products, although analyses such as composition, nutritional value, allergenicity and acute toxicity are typically addressed. In general, three elements are included in the FDA evaluation (32):

1. The safety of the source organism and genetic material used in the transformation process
2. The safety of the gene expression products (usually proteins) resulting from the newly inserted genetic material
3. The safety of the whole plant or food product derived from the plant

Although the current consultation process with the FDA is voluntary, every agricultural biotechnology product commercialized in the U.S. has been evaluated by the FDA and a new proposed rule would require a mandatory premarket notification to the agency (33).

The FDA policy toward the labeling of foods derived from biotechnology is consistent with the existing policy for foods derived by other methods. The FDA's approach to the labeling of foods, including those genetically engineered or otherwise novel, is that the label must be accurate and "material". No labeling is required if the food is not materially different from its conventional counterpart in composition, nutritional value or health effects. There have only been two biotechnology derived foods which have required labeling, a high-laurate canola and a high-oleate soybean product, both of which have oil composition that differs from conventional varieties (25).

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The U.S. regulatory system for the products of agricultural biotechnology is science-based and involves several federal agencies. A comprehensive review of the U.S. regulatory system has been recently published by the National Academy of Science (34). The National Academy of Science had a number of recommendations for improvements in the system but concluded that in general the framework has been operating effectively for over a decade.

Importance of Agricultural Biotechnology for Developing Countries

Gordon Conway, the President of the Rockefeller Foundation, in his book, "The Doubly Green Revolution: Food for All in the Twenty-First Century" has noted that three-quarters of a billion people live in a world where food is plentiful and yet they are hungry and malnourished (35). While in Western Europe and North America average supplies of energy from food exceed 3,500 calories per day, less than two-thirds this amount are consumed in Sub-Saharan Africa and South Asia. Conway argues that the best way to improve crop production in the twenty-first century is to combine conservation of the environment with productivity. A key element in the improvement in productivity is the development of new plant varieties that can deliver higher yields and provide solutions to a variety of challenges such as viruses, fungi, bacteria and insects, as well as drought and salt stresses. Conway suggests that genetic engineering of plants using the tools of agricultural biotechnology can contribute in a very positive way to the development of these new crop varieties.

Florence Wambugu, a plant virologist and former director of the International Service for the Acquisition of Agri-Biotech Applications (ISAAA, AfriCenter), has written very eloquently about the needs of Africa and the role she sees for agricultural biotechnology (36). The average yield of maize in Africa is about 1.7 tons per hectare compared to a global average of 4 tons per hectare. Current biotechnology research is underway to develop a variety of maize that is resistant to the maize streak virus (MSV) that causes losses of up to 100 percent in many parts of the African continent. The project is brokered by ISAAA and involves a collaboration of the Kenya Agricultural Research Institute (KARI), University of Cape Town, the International Centre for Insect Physiology and Ecology in Kenya and the John Innes Centre in the United Kingdom. The sweet-potato feathery mottle virus can reduce yields by 20-80

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percent. After unsuccessfully working for a decade to improve sweet potato production using traditional breeding and agronomy, Wambugu used the tools of agricultural biotechnology to develop a transgenic variety that is resistant to this virus. The sweet potato is currently undergoing field trial testing in Kenya.

In the KwaZulu Natal Province of South Africa, small-scale farmers have enthusiastically planted insect-resistant cotton expressing the *Bacillus thuringiensis* (Bt) insecticidal protein due to the financial benefits (37). Only four farmers took part in the field trials in 1997. The next year 75 farmers planted 200 hectares of Bt cotton. In 1999, this number increased to 410 and 789 hectares followed by 644 farmers and 1250 hectares in 2000. This accounts for approximately 50% of the total hectares planted in cotton in that region.

A recent survey by Huang and colleagues (38) indicates that China is developing the largest plant biotechnology capacity outside of North America. A review of the genetically modified plant field trails indicated that a variety of crops, including rice, wheat, potatoes and peanuts, are currently under investigation. Between 1996 and 2000, China's Office of Genetic Engineering Safety Administration approved 251 cases of genetically modified plants, animals and recombined microorganisms for field trials, environmental releases or commercialization. In China, greater than 90% of the field trials have been directed toward insect and disease resistance. The emergence of a pesticide-resistant bollworm population in the 1980's, resulted in the initiation of research in China to develop a genetically modified Bt cotton plant. In 1997 commercial use of Bt cotton was approved and varieties from publicly funded research institutes and private companies from North America became available to farmers. From only 2000 hectares in 1997, the Bt cotton area had grown to 700,000 hectares in 2000 (35). Furthermore, Bt cotton farmers reduced pesticide use by an average of 13 sprayings (49.9 kg) per hectare per season.

Ismail Serageldin, Chairman of Consultative Group on International Agricultural Research and Vice President of Special Programs of the World Bank, has noted that biotechnology, one of the many tools of agricultural research and development, could contribute to food security in developing countries by helping to promote sustainable agriculture to smallholder farmers (39). For the contribution of agricultural biotechnology to be fully realized, Serageldin has concluded that crop-specific research needs to be integrated into a broader perspective that takes into account sound management of natural resources as well as productivity and profitability of smallholder farmers. In addition, sophisticated research tools such as genetic mapping, molecular markers and agricultural biotechnology need to be focused on the crops and cropping systems that will benefit the peoples of developing countries and their

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environment. To achieve these goals, Serageldin states that there needs to be increased involvement by public research centers in partnership with the private sector to ensure that the technological innovations of agricultural biotechnology are available for research that will directly address the needs of developing countries.

Prospects for the Future

Since the introduction of the first food crop derived through agricultural biotechnology in 1994, remarkable changes have occurred with the adoption of this new technology. With a few exceptions, the crops that have been introduced have been enhanced with agronomic traits such as herbicide tolerance or insect protection that primarily benefit farmers and the environment. Over the next five to ten years, it is anticipated that crops will be introduced with enhanced nutritional food and feed traits that will directly benefit consumers as well as animal producers. In this section, a number of examples of nutritionally enhanced crops that are under development will be described, and the research and regulatory challenges will be reviewed. A more comprehensive presentation of the products of agricultural biotechnology with nutritional and consumer benefits has been published recently (40). These products could be of particular importance in improving global health equity, as noted by Peter Singer and Abdallah S. Daar (41).

Vitamin A deficiency is a major worldwide health problem affecting an estimated 124 million children. Rice (*Oryza sativa*) is a major staple food in people's diet in at least 26 countries, including the highly populated areas of Asia, Africa and Latin America. Removal of the oil-rich aleurone layer of rice to prevent rancidity upon storage results in the edible endosperm lacking several essential nutrients including the precursor of vitamin A, β-carotene. Rice enhanced with β-carotene using the tools of agricultural biotechnology was developed by Ingo Potrykus and colleagues (23) as an approach to provide vitamin A for deficient populations. The resulting rice is golden due to the presence of increase levels of β-carotene. Some analyses indicate that the levels of β-carotene that would be contributed to the diet through "golden rice" are insufficient to alleviate vitamin A deficiency completely (42). However another evaluation (40) has indicated that substitution of "golden rice" for regular rice in the diet of populations with low intake of vitamin A could make a beneficial difference in reducing the symptoms resulting from the deficiency.

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Some oil in the diet is needed to promote the adsorption of vitamin A since it is a fat-soluble vitamin. Recent biotechnology research has successfully elevated the levels of carotenoids, primarily α- and β-carotene, in a high oil crop, canola (*Brassica napus*) (40). The resulting oil from this canola seed contained about 2000 μg of carotenoids per gram, whereas the next richest source of carotenoids, red palm oil, contains about 600 μg/gm. Currently, a joint research project has been established among four organizations [Tata Energy Research Institute, a not-for-profit Indian research institute in Delhi, Michigan State University's Agricultural Biotechnology Support Project and the Monsanto Company, with support from the U. S. Agency for International Development (USAID)] to use the technology developed for canola to produce a "golden mustard" oil that is high in β-carotene. Mustard oil is a staple food product in India, so a high β-carotene mustard oil would be readily assimilated into the Indian diet. Like all products developed using agricultural biotechnology the golden mustard oil will be evaluated for safety and efficacy and must obtain regulatory clearances prior to introduction on the market.

Palm oil is widely used in tropical regions such as Central and South America, West Africa and Malaysia. The oil is rich in palmitic acid, a saturated fatty acid, that is less desirable than unsaturated fatty acids, due to the link between saturated fat in the diet and increased risk of cardiovascular disease. Research is underway in Malaysia to increase the conversion of palmitic acid to its unsaturated analogue, oleic acid, by promoting the expression of the gene for the enzyme, β-keto acyl ACP-synthase II using the tools of agricultural biotechnology (43). Because palm oil is rich in carotenoids, tocopherols, tocotrienols and sterols, the enrichment with oleic acid would further enhance its favorable nutritional properties and promote acceptance in non-tropical populations.

Iron is another essential nutrient that is deficient in the diets of many people of both the developed and developing countries. Iron deficiency is sufficiently severe in parts of the developing world to result in widespread anemia. The problem arises due to the almost exclusive reliance on plant foods in the diet that are low in bioavailable iron and may have levels of phytate that interfere with iron absorption. Goto and coworkers have reported the successful insertion of the soybean gene for ferritin, the iron storage protein, into rice (44). Ferritin stably accumulated in the rice endosperm resulted in a threefold increase in iron (38.1 ± 4.5 μg iron/gm dry weight of tissue) in the genetically modified rice when compared to conventional rice (11.2 ± μg iron/gm). These results suggest that "ferritin rice" may be useful as a supplement to the human diet. Additional efforts have been directed toward enhancing the uptake of iron. Lucca and

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colleagues have inserted a ferritin gene into rice as well as a gene that codes for a metallothionein protein to promote iron absorption (45). Also these researchers inserted the gene for phytase so that the interference of iron absorption by phytate would be reduced.

Many other product ideas are in the research stage including plants tolerant to drought, salt and low temperatures (46) and other plants that could be useful as edible vaccines (47). The agricultural biotechnology protocols useful in gene discovery and product development have been described in two recent reviews (48, 49).

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Concluding Thoughts

The past 150 years have been marked by stunning technical breakthroughs in our understanding of the basic biochemical, genetic and physiological processes in living systems, particularly plants. This information has resulted in new products that have provided farmers with new tools to enhance the productivity and decrease the environmental impact of agricultural practices. Exciting new products are under development that could contribute to an improvement in the nutritional health in many countries in the developing world.

Questions have been raised about the safety of the food supply derived from these products (22), their effects on non-target organisms (20, 21) and the potential for unintended effects (50). The scientific evidence regarding the important topic of safety of existing products derived through agricultural biotechnology for humans, animals and the environment will be addressed in subsequent chapters of this book. Scientific investigations continue to be conducted and the new products, such as those designed for enhanced nutrition, must be evaluated for safety prior to introduction into the marketplace. In future years, the acceptance of these studies by governmental agencies and consumers worldwide will determine if the promise of agricultural biotechnology will be fully realized (51, 52, 53).

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Chapter 2

The Future of Agrobiotechnology: Implications for National Competitiveness

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Agrobiotechnology was first commercialized in the mid-1990s and was quickly embraced by farmers. Consumers in some parts of the world, however, have been skeptical towards the technology. This apparent dissonance between technology providers and users on the one hand, and some consumer quarters on the other, has led to conflicting views on the future of agrobiotechnology. In this paper, I look at emerging trends in some key factors—technical innovation, institutions, and markets—and discuss their implications for the future of agrobiotechnology and consequent national competitiveness.

Introduction

By now the story of agrobiotechnology is well known. After almost twenty years in the lab and the experimental fields, agrobiotechnology was first commercialized in the mid-1990 and was quickly embraced by farmers in key agricultural producing countries *(1)*. Consumers in Europe and some other parts of the world, however, have been skeptical and often combative towards the new technology (*2*). This apparent dissonance between technology providers and

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users on the one hand, and some consumer quarters on the other, has led some to speculate that agrobiotechnology is heading the way of nuclear energy—a promising technology but ultimately one with unfulfilled expectations (*3*). Interestingly, such gloomy predictions have coincided with others that have hailed agriculture as the primary beneficiary of the emerging genomics revolution (*4*). Will agrobiotechnology live up to the high expectations set out by its purveyors for over a quarter of a century?

Although it may be difficult to predict the future trajectories of agrobiotechnology, the factors that will shape them are more or less understood. Technical prospects, institutions, and markets will shape the future of agrobiotechnology. In this paper, I look at emerging trends in these key factors and discuss their implications for the future of agrobiotechnology and for national competitiveness.

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Technical Innovation, Economic Value, and Competitiveness

Fundamental scientific discoveries and technical breakthroughs can profoundly affect economic growth and competitiveness (*5*). When such radical innovations link together and reinforce each other through further discoveries, they can cause far-reaching structural, social, and economic changes and affect multiple sectors of the economy or give rise to new ones (*6*). Biotechnology has the makings of just such a technology platform. It is a collection of diverse and reinforcing enabling technologies with a wide range of applications in agriculture, forestry, food processing, waste management, pollution control, chemicals, raw materials, energy, cosmetics, pharmaceuticals, and probably other sectors that will become apparent in the future (*7*).

In agriculture, first-generation biotechnology products have been crops with improved agronomic properties, such as herbicide tolerance and resistance to particular insect pests. Technologically, first-generation agrobiotechnologies are rather elementary—scientific "easy pickings." They require expression of a novel gene or over-expression of a target enzyme in the case of herbicide tolerance. Such single gene events cause discrete changes in phenotypes; accordingly, they have been relatively easy to isolate and map. Product development is also accelerated by the availability of analytics that assess performance at early developmental stages. Phenotypes of herbicide tolerance, for example, appear in transformed single cells, while other traits, such as higher yields or improved nutritional profiles, can be fully evaluated only in finished inbred lines (*8*).

Second-generation bioengineered crops with enhanced quality traits, such as corn with modified oil and more balanced amino acid ratios (e.g., elevated lysine

content) are being developed, targeting the feed, edible oils, and food markets. Industrial and pharmaceutical uses are also targeted through bioengineered plants that yield improved industrial feedstocks (e.g., oils, starches, and other polymers) but also enhanced nutritional and pharmaceutical agents or neutraceuticals (*9*).

Second-generation agrobiotechnologies are technically more complex than first-generation traits. They are associated with many genes or gene complexes acting in concert (*10*). A direct implication is that to advance second-generation agrobiotechnologies systematically, researchers must coordinate expression of several genes over the growing period of a crop. In this direction, plant genomics, proteomics and bioinformatics will play an integral part in providing solutions.

The emergence of genomics and bioinformatics in the 1990s has radically changed the research paradigm in biotechnology and has elevated expectations about the rate of discovery and product development in agrobiotechnology. In the 1970s and 1980s, the focus of agrobiotechnology was on the isolation of commercially useful genes and on methods of inserting and expressing such genes in commercially relevant species. Genes will be in excess over the next several years. The new research paradigm is the study of complex biochemical and physiological problems as an integrated system, rather than on a gene-by-gene basis. The discovery that large blocks of genetic material are collinear across many species has opened up new discovery venues. Various genome projects of microbes, plants, and animals from numerous taxa enable inferences among species. The best place to look for a gene in a particular species can be at a different species altogether (*10*). As biotech research has moved away from *in vivo* and *in vitro* systems to *in silico* and *ex machina,* scientific discovery has reached unprecedented rates.

Since much of the scientific and technical knowledge of agrobiotechnology lies ahead, so does its economic potential. Yet, first-generation agrobiotechnology products provide a glimpse of such potential. Herbicide tolerant and insect resistant crops have created significant on-farm economic value through diminished average costs. Unit cost reductions result from reduced insecticide and herbicide use, managerial labor savings, improved risk management in pest control, and increased yields (*11,12,13*). In response to such profit opportunities, over 50% of the US corn, soybean, and cotton acres were planted with bioengineered crops within five years of commercial introduction. Similar adoption rates for bioengineered crops have been observed in Canada and Argentina (*1*).

Second-generation agrobiotechnologies are technologically more complex, but also economically more intriguing, as they create economic value through product differentiation. Crops with bundles of attributes highly valued by particular end-users present possibilities for increasingly sophisticated market

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segmentation, leading to expansion of market value (*14*). Industrial and pharmaceutical applications create entirely new economic possibilities and value (*15*).

Staking claim on current and future economic gains from agrobiotechnology could therefore have significant long-term competitiveness implications. By all conventional metrics, the US is a leader in agrobiotechnology research and product development. US corporations and public research institutes or universities own over 90% of the US agrobiotechnology patents and all top twenty-five patents, as defined by agrobiotechnology patent citations (*14*). US ownership of European agrobiotechnology patents exceeds 50%. The US has also dominated product experimentation and development. US firms and research organizations have experimented with a larger number of crops and traits than any other nation. By the end of 2000, the US was the site of almost 50% of all bioengineered crop field trials performed globally. The US was also the site of over 68% of all acreage devoted to agrobiotechnology at a global scale in 2001 (*1*).

Will the US be able to translate its scientific and technical leadership into sustained market leadership and international competitiveness? This is yet to be determined, but institutions and public sentiment toward the technology will play a key role.

Institutional Change and Public Attitudes

Diffusion of radical technical innovation requires institutional and organizational change and the creation of an appropriate infrastructure (*6*).[1] Institutional change is typically slower than technical change itself. In the case of agrobiotechnology, science and technology have developed rather slowly over a twenty-five year period. They have, however, easily outpaced institutional change, which has moved with glacial speed.

Discussions of appropriate regulatory norms for agrobiotechnology date back to the early 1980s. In 1986, the OECD Council Recommendation was that risks of bioengineered organisms were expected to be the same as those of conventional ones, and could be assessed in similar ways (*17*). This notion of "substantial equivalence," however, has been mirrored in few regulatory regimes around the world. In the US, the Food and Drug Administration (FDA) adopted such a regulatory approach, whereby products of agrobiotechnology are assessed within existing frameworks for safety and nutritional fitness. A similar approach

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[1] Institutions here are defined, *a la* North, as societal rules that reduce uncertainty and shape human interaction (*16*). Of all institutions, I focus here on regulatory norms due to their relative importance.

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was adopted in Canada. However, most regulation, including EPA's framework regulating environmental releases in the US, has focused on the process of biotechnology rather than its products.

In the European Union (EU), a process-specific regulatory framework was also adopted early on, thereby regulating biotechnology by newly installed institutions, starting with Directives 90/219 and 90/220 on the contained and deliberate release of "Genetically Modified Organisms" (GMOs) in 1990. Since that time, the regulatory framework in the EU has been work in progress, frequently revised and reshaped by different legislative bodies.[2] The 1997 revision of Directive 90/220 was particularly important, as it installed mandatory labeling for "GMOs." Subsequent revisions of 90/220 introduced, among other things, "ethical considerations" in the regulation of agrobiotechnology and a *de facto* moratorium on new product approvals in the EU, which was still in place by the end of 2001.

Horizontal legislation in the EU has been complemented by vertical regulations for specific product risks that have also been subject to continuous revisions. Regulation 257/97 on novel foods made labeling of foods produced from genetically modified (GM) ingredients mandatory in 1997. However, it was not until a year later (regulation 1139/98) that presence of foreign DNA or newly expressed proteins in foods was made the criterion for labeling. A standard was finally established in 1999 when the threshold of foreign DNA triggering mandatory labeling was set at 1%. Further revisions requiring mandatory labeling for food additives and flavorings in processed foods came into effect in April 2000 (regulations 49/2000 and 50/2000). In July of 2001, the European Commission introduced a new legislative proposal for regulating GMOs, GM food, and GM feed. If adopted, this latest legislation will require labeling and traceability of all foods, food additives, feeds, and feed additives produced from bioengineered commodities.

Interestingly, regulation and public opinion towards agrobiotechnology have co-evolved. In the US and Canada, the regulatory regime has been largely unchanged, paralleled by relatively stable and positive public attitudes towards agrobiotechnology (*2*). In the EU, increased regulatory oversight has coincided with increasingly negative public attitudes towards agrobiotechnology and diminishing trust in public authorities and regulatory agencies (*2*). It appears that increasingly negative public attitudes have led to tighter regulation in the EU (*18*). However, such reactionary regulation has satisfied few. A constantly changing regulatory environment has been viewed as an institutional vacuum and has been filled by private initiative. Certain EU food retailers and food

[2] Several Directive Generals are involved with shaping biotechnology regulation in the EU. They include: DG III—regulating novel foods, DG VI—regulating feeds and seed, and DG XI—regulating the deliberate releases of GMOs.

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manufacturers have moved to establish voluntary standards and labels relevant to their market situation. With almost no exceptions, voluntary standards have been set to zero or near-zero tolerance for biotechnology products, leading to "non-GM" or "GM-free" claims.

Zero tolerance standards in the EU have translated into reformulation of processed foods to remove biotechnology products or their derivatives and into identity-preserved or traceable supply chains to assure the absence of such products. In the highly concentrated EU food markets, voluntary "GM-free" or "non-GM" labels have quickly become the standard, making mandated labels and thresholds practically irrelevant. [3]

Other countries have also implemented mandatory labeling. Japan and South Korea have introduced mandatory labeling for food products that contain over 5% and 2% of GM ingredients, respectively. Mandatory labeling rules in both countries, however, explicitly exclude feed, oils, and highly processed foods from labeling requirements. In Australia and New Zealand, mandatory labeling is required for foods, processed foods, fruits, and vegetables that contain more than 1% of GM material. Meanwhile, Canada and the US recently provided guidelines for voluntary labeling. Under such conditions, the regulatory regime across the globe will likely remain fragmented, as regulatory standards will tend to vary significantly from one country to another.

International conventions with standard-setting authority are unlikely to provide much assistance with homogenizing fragmented regulation in the short run. Various regulatory rules are currently debated at different multilateral organizations. Discussions on the labeling standards for agrobiotechnology products began in the UN Codex Alimentarious in 1993. After eight years of deliberations, however, consensus among members on a common standard remains elusive (*19*). Even basic elements of what is to be labeled and when a label may be necessary remain unresolved. As of May 2001, the Codex working group had agreed only on some very basic definitions; progress on the key elements of a standard remained under discussion (*20*). The UN Convention on Biological Diversity Biosafety Protocol continues to be debated, and there is significant controversy on its scope, especially in reference to the "precautionary principle." Recently, the FAO International Plant Protection Committee began

[3] Food retailing is highly concentrated in many parts of the world, including the EU. The top five retailers in the UK, for instance, command some 80% market share. For France and Germany, CR5s are 81% and 64%, respectively. Decisions under such market structures are highly centralized, and competitive matching can quickly lead to a common standard. In the UK, for instance, all major chains including Tesco, Sainsbury, Asda, Somerfield, Safeway, and Marks and Spencer adopted "non-GMO" standards within months from the time smallish Iceland announced its intent to adopt such a voluntary standard in 1998.

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discussions on the safety of biotechnology crops, further complicating the issue of which international organization regulates the release and safety of agrobiotechnology products.

In all, institutional change relevant to agrobiotechnology has been slow and has failed to establish commonly acceptable standards amenable to decision making. Furthermore, the introduction of the precautionary principle and "ethical standards" as regulatory norms have signaled a fundamental departure in regulatory philosophy, with potentially far-reaching implications. As such norms continue to be debated, institutional change will slow down even further. It is likely that, at least in the short run, firm initiative and voluntary standards, along with a fragmented regulatory regime, will mediate uncertain market conditions.

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Short Term Market Realities: Muddling Through

Fragmented regulation and lack of standards could encourage market segmentation. In practice, two market segments have emerged—one where agrobiotechnology products continue to be traded without restrictions and a "non-GM" segment. Key commodity markets affected by agrobiotechnology (i.e., corn, soybeans, and canola), however, have shown little strain by such segmentation, as supply and demand shifts have been limited and gradual (*21*). Tables I-III present production, consumption, and trade flows for a group of ten countries/regions which will play a key role in the future trajectories of agrobiotechnology. These countries dominate production, consumption, and the global trade of agricultural commodities and processed foods. They have also led in the development of agrobiotechnology, as well as the debates on its labeling and regulation.

With the exception of Mexico and Taiwan, all major importing countries have experienced some degree of negative consumer response towards agrobiotechnology and have installed mandatory labeling regulations. Consumers in all major exporting countries have been more accepting of the technology, and domestic markets have been largely undisturbed. As illustrated in Tables I through III, domestic consumption in these countries dominates exports and hence demand shifts have been limited. Demand shifts have also been minimized as feed use dominates both the domestic and international markets for corn, soybeans, and canola. Feed markets in all major markets, including the EU, have remained largely unaffected by existing regulation.[4]

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[4] Most feedstuffs (including corn gluten and soybean meal) imported by EU countries in large amounts are not covered by existing mandatory labeling laws and hence remain unregulated. In all other countries with mandatory labeling regulations, feedstuffs are explicitly excluded from labeling requirments.

Table I. Production Trade Flows of Corn for Selected Countries (1996-2001 avg.)

Country	*Production*	*Net Imports*	*Net Exports*	*Domestic Consumption*	*Consumed as Feed*
United States	241.4		47.1	193.9	75%
China	117.8		5.6	116.9	76%
Argentina	16.1		10.7	5.4	70%
Brazil	34.5		0.01	34.6	86%
EU	37.3	2.1		39.2	78%
Mexico	18.3	4.9		23.3	35%
Japan	0.0	16.1		16.1	74%
Taiwan	0.1	4.9		5.0	95%
South Korea	0.1	7.9		8.0	78%
Canada	7.8	1.2		9.1	79%
Percent of World	80%	62%	94%	76%	81%

NOTE: Units are in million metric tons

SOURCE: USDA, ERS PS&D database

Table II. Production Trade Flows of Soybeans for Selected Countries (1996-2001 avg.)

Country	*Production*	*Net Imports*	*Net Exports*	*Domestic Consumption*	*Consumed as Food*
United States	73.2		32.7	39.8	18%
China	14.6	10.0		23.4	40%
Argentina	20.9		19.5	1.3	11%
Brazil	34.5		22.2	12.4	24%
EU	1.3	28.4		29.6	6%
Mexico	0.1	4.1		4.1	19%
Japan	0.2	5.7		5.6	30%
Taiwan	0.0	2.4		2.4	28%
South Korea	0.1	2.6		2.7	27%
Canada	2.5	0.3		2.7	10%
Percent of World	92%	70%	92%	78%	72%

NOTE: Units are in million metric tons

SOURCE: USDA, ERS PS&D database

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Table III. Production Trade Flows of Canola for Selected Countries (1996-2001 avg.)

Country	*Production*	*Net Imports*	*Net Exports*	*Domestic Consumption*	*Meal Used as Feed*
United States	0.6	1.5		2.2	100%
China	10.0	1.2		10.7	33%
Argentina	0.0			0.0	
Brazil	0.0			0.0	
EU	9.1		0.1	9.0	100%
Mexico	0.0	0.9		0.8	100%
Japan	0.0	2.2		2.1	66%
Taiwan	0.0	0.1		0.1	100%
South Korea	0.0	0.4		0.4	89%
Canada	6.6		5.2	1.6	100%
Percent of World	73%	92%	82%	77%	74%

NOTE: Units are in million metric tons

SOURCE: USDA, ERS PS&D database

If pending proposals for GM feed labeling and traceability in the EU were adopted, however, they could affect international trade. As evident from Tables I and III, in the international markets for corn and canola, the EU is a minor player and has little impact on trade.[5] In the soybean market, however, EU is the primary import market and significant shifts can have meaningful impacts on trade flows (Table II). If mandatory labeling and traceability for feedstuffs produced from bioengineered commodities were finally implemented, identity preservation systems would be required to supply the EU market.

Identity preservation implies additional costs. Farmers must be compensated for the direct costs in segregation and for foregone profits from not using bioengineered crops. Similarly, grain handlers must be compensated for direct logistical costs for segregating, testing, and certifying crops from field to market. Identity preservation costs at the grain handler level can be substantial, especially under strict purity standards (*22*). They are much higher when opportunity costs are added to account for capacity underutilization, as well as foregone profits from spread opportunities and other value added activities (*23*).

[5] The EU has little impact on commodity corn. It is, however, the largest buyer of corn gluten, a by-product used as animal feed, in the world. Supplies originate mainly from the US; at almost $500 million it is a significant market.

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As such costs are borne by all food merchants, they will ultimately be passed to the consumer.

In all, with the EU soybean market being a possible exception, no major short-term market disruptions should be expected. Despite the existing regulatory muddle, international markets have continued to function without problems.

Long-Term Prospects for Agrobiotechnology

Short-term market difficulties have clashed with the long-term visions of a scientific revolution that could transform the agrifood sector or the economy as a whole. How are we to understand today the long-term prospects of agrobiotechnology? An indicator of such long-term prospects is current public and private investments in agrobiotechnology R&D, as they reflect expectations of key stakeholders.

Initial evidence suggests that significant private and public investments are being directed towards agrobiotechnology R&D. Table IV presents selected research agreements in agricultural genomics and bioinformatics signed over the last four years. More than one hundred such private-private research agreements were announced in this period, involving significant funding commitment. Most have been signed between large, established firms and knowledge-based biotechnology startups, indicating the beginning of a new lifecycle in agrobiotechnology (*24*). Furthermore, in addition to established firms with lengthy commitment in agrobiotechnology (e.g., Aventis, Monsanto, and Novartis), new entrants have also invested (e.g., BASF) while others have begun to leverage the technology in more traditional sectors, such as chemicals (e.g., Bayer).

Government supported R&D investment in agrobiotechnology has also continued uninterrupted. If anything, it has recently increased in many parts of the world, including in countries that have installed significant regulatory restrictions on agrobiotechnology in their home markets. The average annual public investment for agricultural and food biotechnology R&D in the EU was approximately $500 million over the period 1994-98 (*25*). Over 80% of such investment was concentrated in just three countries—France, Germany, and the UK. Significant additional funding was provided for basic research, including the development of genomic and bioinformatics methods and tools. Japan has also increased its investment in agrobiotechnology in recent years. In 2000 alone, Japan spent $260 million in agrobiotechnology research—$216 through MAFF and $44 million for the rice genome project.

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Table IV. Research Agreements in Agrobiotechnology (1996-2000)

Company Name	*Company Name*	*Research Area*	*Year*	*Contract Value*
AgrEvo	GeneLogic	Disease resistance	1998	$45million
AgrEvo	Netgenics	Bioinformatics	1999	NA
American Cyanamid	Hyseq	Genomics	1999	$60 million
Aventis	Lynx	Functional genomics	1999	NA
BASF	SunGene	Plant biotechnology	1999	NA
BASF	Metanomics	Plant biotechnology	1999	NA
BASF	Incyte	Genomics	1996	NA
Bayer	Arqule	Library screening	1999	$30million
Bayer	Exelixis	Chemical screening	2000	$200 million
Bayer	Paradigm Genetics	Chemical screening	1998	$40 million
Bayer	Lion Bioscience	Genomics	2000	25 million
Ceres	Genset	Gene sequencing	1999	NA
Dow Agro	BioSource Technlogies	Functional genomics	1997	NA
Dow Agro	Exelixis	Chemical screening	2000	NA
DuPont	Maxygen	Novel Genes	1999	NA
DuPont	Lynx	Gene identification	1998	$60 million
FMC	Xenova	Novel insecticides	1998	NA
Hitachi	Myriad Genetics	Proteomics	2000	$26 million
Monsanto	Paradigm Genetics	Functional genomics	1999	NA
Monsanto	Genetracer	Genomics	1997	NA
Monsanto	Millenium	Plant genomics	1997	$218 million
Novartis	Myriad Genetics	Cereal genomics	1999	$34 million
Novartis Agribus	Diversa	Novel enzymes	1999	NA
Novartis Institute	Invitrogen	Functional genomics	1999	NA
Novartis Institute	EraGen	Genotyping	2000	NA
Pioneer	CuraGen	Genomics	1998	NA
Pioneer	Maxygen	Gene performance	1999	NA
Pioneer	Oxford GlycoScience	Protein analysis	1998	NA
RhoBio	Celera AgGen	Gene discovery	1999	NA
RhoBio	CSIRO	Gene expression	1999	NA
Rhone Poulenc	Agritope	Functional genomics	1999	$20 million
Zeneca	John Innes Centre	Wheat genomics	1998	NA
Zeneca	Maxygen	Input/output traits	1999	$25 million

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These expenditures do not include other significant investments in infrastructure, public subsidies for biotechnology startups, and subsidies for research on the safety of agrobiotechnology. South Korea has also committed significant public funds for agrobiotechnology research, including $173 million through the Ministry of Agriculture for the year 2000 alone. For China, information on the size of public investment is sketchier. Private sector consultants estimate the Chinese public investment in plant biotechnology at approximately $300 million in 2000. Irrespectively, the fast-expanding capability of China in agrobiotechnology is unmistakable. In the last few years, it has developed and field-tested numerous transgenic crops (e.g., rice, wheat, corn, and various vegetables) with novel agronomic traits and quality improvements (*26*). Furthermore, it has recently expanded its capability in genomics (e.g., in rice and other cereals), mapping, identifying, and cloning useful genes, and other advanced techniques (*26*).

Public investments in agrobiotechnology across these and other countries are not occasional. They are components of coherent multiyear public programs with strategic intent. In the EU, both supranational framework programs (e.g., BRIDGE, ÉCLAIR, and AIR) and national programs (e.g., BioAvenir and Genoplante in France and Biotechnology 2000 in Germany) have been put in place. Japan has initiated the so-called "Millenium Program" targeting the creation of 1000 biotechnology firms by year 2010. In Korea, funding is provided through the 14-year/$20 billion "Biotech 2000" program, which started in 1994, with agrobiotechnology playing a central role.

Public investments in various countries closely imitate the US biotechnology model by encouraging the creation of research startups, biotechnology networks, science parks, and private-public research agreements. This model has been credited for the leadership position of the US in biotechnology (*27, 28*). Through its 5th PCRDT Programme, the EU provides funding for encouraging bio-entrepreneurship and the creation of R&D networks. National programs like "Bio-Regio" in Germany and the "Genopole" in France pursue similar goals and the creation of biotechnology clusters (*29*). The Chinese government has pledged some $2 billion for the creation of a "Chinese Biotech Valley" in the Yunnan area. Japan and Korea have gone beyond subsidizing bio-entrepreneurship by introducing legislation similar to the US Bayh-Dole Act in order to encourage private-public collaborative efforts.

Judging from the levels of private and public R&D investment across the globe and the strategies that are being implemented, the overriding conclusion is that agrobiotechnology remains a strategic innovation in all quarters. The genomics revolution has created a new research paradigm. It has opened up new possibilities for product development and has presented new entrants and latecomers with possibilities to close the technical gap with the US. Under such

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circumstances, heightened public agrobiotechnology investments are rational. They are also consistent with strategic investment theories (*30*).

A key question, then, is whether public investments in agrobiotechnology research and capacity building across the globe will succeed in closing the technical gap between the US and other nations. In this direction, the evidence is mixed. In some cases, international investments seem to be paying off. In the EU, for instance, the number of biotechnology startups has increased drastically in recent years—a definite indicator of improving industrial dynamism (*31*). At the same time, over 80% of all research agreements signed around the globe in cutting-edge agrobiotechnologies, including those in Table IV, have been directed to US startups—a definite indicator of technological leadership. The suspicion is that most of the EU startups are service-oriented companies rather than cutting-edge, high-risk research ventures (*32*). Interestingly, the flow of R&D investments in US startups contributes towards new skills and know-how, precisely the externalities that reinforce technological leadership.

Concluding Comments

Public and private investment behavior in recent years provides a clear signal that agrobiotechnology remains a strategic innovation around the globe. Lack of clarity in national and international institutions that regulate agrobiotechnology around the world has hampered investments and product commercialization and will continue to do so for some time.

Given the early, critical stage in the genomics revolution and the path dependent and cumulative nature of innovation, short-term market and institutional realities can and will shape the future of agrobiotechnology. In this context, countries and regions facing prolonged institutional and market uncertainties could significantly obstruct the long-term development of their biotechnology and related industries. The US is a leader in agrobiotechnology research and product development. With a large domestic market, relatively coherent institutional framework, flexible capital markets, and a dynamic biotechnology industry, the US is well positioned to build on its current technical leadership and its competitive advantage for the future.

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Chapter 3

Insecticidal *Bacillus thurgingiensis* Plants versus Chemical Insecticides

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Genetically engineered crop plants express insecticidal proteins from *Bacillus thuringiensis* (Bt) are compared to conventional insect control practices using chemical insecticides in terms of ease of use, efficacy, cost and adoption. These new varieties have been commercialized and adopted for field corn and cotton. Bt varieties of sweet corn and potatoes have been approved but not adopted by growers. Several other crops have been engineered to express Bt proteins, including sweet corn, potatoes, soybeans and peanuts. Corn growers have increased yields while cotton growers have reduced insecticide use by 3 million pounds annually. Projected benefits are presented for crops in development.

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Genetically engineered crops have been rapidly adopted by U.S. growers due to the potential for reducing production costs and increasing yields. Insect resistant crops were developed through the insertion of genes derived from the soil bacterium *Bacillus thuringiensis* (Bt) that encode proteins toxic to specific groups of insects. The crystalline proteins are activated by digestive enzymes in the insect gut, binding to specific receptors which results in ruptures of the intestinal lining. The insect stops feeding almost immediately, and dies eventually from starvation.

Toxins isolated from *Bt* varieties have been commercialized and applied as foliar insecticides for more than 40 years (*1*). They dominate the biopesticides market and are widely used throughout the U.S., particularly in organic production. In 1981, a *Bt* gene encoding a Cry protein was cloned and successfully transferred to and expressed in another organism, the bacterium *Escherichia coli* (2). Within ten years, tomato, tobacco and cotton plants had been transformed to express *Bt* Cry proteins (*3,4,5*), and *Bt* corn and potato plants were developed soon thereafter (*6,7*).

The potential benefits of genetically engineered crops depend on their efficacy and cost relative to alternative practices. Each of these crops offers a distinct set of benefits over conventional insect management programs depending on particular pest control needs and available alternatives. Here, a brief overview of the impacts of the introduction of Bt varieties of field corn for corn borer, cotton, as well as projected impacts of Bt varieties of sweet corn, potatoes, field corn for rootworm, soybean and peanuts is provided. Understanding the reasons why growers are adopting, and might be likely to expand their adoption of, genetically engineered crops is critical in an evaluation of the impact of the technology on U.S. agriculture.

Field Corn for Corn Borer

Bt field corn varieties were introduced in 1996 and have been adopted by a large segment of corn growers, rising to a high of 26% of total corn acreage by 1999, and falling to 19% for the 2000 and 2001 growing seasons. Adoption is expected to climb to 24% for the 2002 growing season (Figure 1). Currently marketed varieties were engineered to express the Cry1A(b) Bt protein, which is active against certain lepidopteran, or caterpillar insects. The primary target pest of these Bt field corn varieties is the European corn borer (ECB). Bt field corn also provides control of southwestern corn borer, fall armyworm, corn ear worms, and stalk borers. The primary impact of the introduction of Bt field corn varieties has been increased yields associated with improved control of corn borers.

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The ECB is considered the second most important insect pest of corn in most regions, after soil inhabiting species such as rootworm (*11*). European Corn Borer larvae move into the whorl of the corn plant shortly after hatching, where they feed on leaves, which results in small holes and patchy areas lacking leaf tissue. Eventually the larvae crawl out of the whorls and down the side of the plant to burrow into the stalk, where they continue to feed until they pupate.

In order to be effective, insecticide treatments must be made during the 2 to 3 day period after ECB eggs hatch and before the larvae bore into the corn stalk, where they are protected from the treatment. Egg hatch may occur over a period of several weeks, so a treatment made at any one point in time will only control a portion of the pest population. In addition, with highly variable and difficult to predict pest pressure from year to year, growers in many areas would not expect pest pressure to be high enough in an average year to require treatment. Therefore, many do not incur the costs of scouting to determine whether pest pressure is high enough to make an insecticide treatment cost effective. A 1985 USDA survey indicated that 4% of the Corn Belt's acreage was treated with insecticides for ECB control (*11*).

Largely uncontrolled in most corn growing areas of the US, ECB has regularly reduced yields. USDA estimates of the annual losses due to ECB varied from a low of 33 million bushels to over 300 million bushels per year (*12*). Annual losses in production and management costs associated with ECB have been estimated at $1 billion (*13*).

Growers who adopt Bt corn have gained control of the ECB, and have therefore experienced increased yields. Field performance tests showed that Bt corn sustained 95% less ECB damage compared to the control plants (*6*). In 1997, we estimate that growers who planted Bt corn had increased yields of nearly 12 bushels an acre on average. In 1998 and 1999, years with lower ECB pest pressure, the average yield advantage for adopters of Bt corn was 3-4 bushels per acre. Low pest pressure in the past two growing seasons, combined with low corn prices are believed to explain the slight decline in adoption of Bt corn in 2000 and 2001 (*14*).

Reductions in insecticide use have been observed since the introduction of Bt corn varieties, though these reductions are modest due to low levels of insecticide use for ECB prior to the introduction of Bt corn. Five insecticides are recommended for ECB control: Bt (foliar spray), chlorpyrifos, lambdacyhalothrin, methyl parathion, and permethrin. These insecticides are typically used to control several pests, which makes attributing any observed changes in their use problematic. Further, lambdacyhalothrin was introduced in

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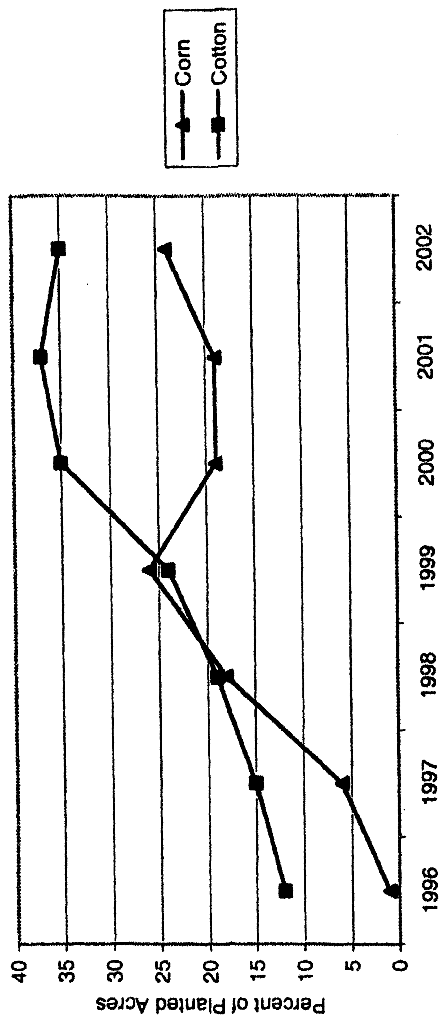

Figure 1. Adoption of Bt Corn and Cotton in the U.S. (1, 10, 11)

1996, primarily for the treatment of cutworm, likely displacing the use of other insecticides towards that target pest. The aggregate reduction between 1995 and 2000 in the percent acreage treated with the remaining four insecticides was 6% (*15*). If one half of this observed decline is due to the introduction of lambdacyhalothrin, the remaining half could be attributed to the introduction of Bt corn, implying over 1 million fewer acres treated for ECB.

Cotton

Bt cotton varieties were also introduced in 1996. Adoption has increased steadily since then, reaching over 37% of U.S. planted cotton acreage by 2001 (Figure 1). Currently available Bt cotton varieties control three primary target pests: cotton bollworm and tobacco budworm in the south and southeast, and pink bollworm in the southwest. The popularity of Bt cotton was at least partially driven by the decreased effectiveness of some conventional insecticides. In Alabama especially, growers faced devastating yield losses of nearly 30% on average due to tobacco budworm and cotton bollworm in 1995, the year before the introduction of Bt cotton varieties, despite making nearly 7 insecticide treatments targeted to these pests. The primary impact of the introduction of Bt cotton has been decreased insecticide use.

The cotton bollworm and tobacco budworm are among the most damaging insect pests of cotton. The principal insect pests of cotton are those that attack the bolls or the flower buds that precede them. While damage due to any particular pest varies from year to year, cotton bollworm/tobacco budworm, boll weevil, and plant bugs have dominated the list of the most injurious pests of cotton for the last 20 years (*16*). Each year, cotton growers apply pesticides to control a variety of insect pests that would otherwise reduce yields. In 2000, 80% of U.S. upland cotton acreage was treated with insecticides. In several states, over 90% of the acreage is normally treated (*17*). Despite the use of insecticides, cotton growers still experience yield losses due to insect pests. In 1995, the year before Bt cotton varieties were introduced, it is estimated that an average of 2.4 insecticide applications were made to control bollworm/budworm across all cotton producing states, and that a 4% yield loss was incurred due to these two pests. Several of the commonly used insecticides for bollworm/budworm control are pyrethroids, which have been preferred due to their low cost. However, the development of pyrethroid-resistant budworm populations has diminished their usefulness in recent years. The severe budworm problems experienced by Alabama growers in 1995 were due to the ineffectiveness of the pyrethroids in face of high levels of budworm resistance.

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Cotton growers who have adopted Bt varieties have realized increased yields, and savings on insect control costs in some years, depending on pest pressure. Bt cotton was shown to provide 95% control of tobacco budworm, 70-90% control of cotton bollworm and 99% control of pink bollworm (*18*). Survey results from over 100 side by side comparisons of Bt and conventional cotton fields across the southern states showed that in 1998, Bt cotton growers saved about $15/acre, while in 1999, a year with lower pest pressure, Bt cotton growers paid about $14/acre more, including the technology fee of approximately $32/acre. However, in both years, Bt cotton fields had higher yields and higher net returns, $40/acre in 1998 and $25/acre in 1999. Increased yields in 1999 outweighed higher insect control costs.

The total amount of insecticides used to control cotton bollworm, tobacco budworm and pink bollworm has decreased since 1995. Twelve insecticides are recommended for the control of these three insect pests. The use of these 12 insecticides in six major cotton states in 1995 compared to 2000 is shown in Table 2. The use of 3 of these insecticides increased, likely due to substitution of these new products for some of the older products used for the same target pests. These increases are overwhelmed, however, by much greater reductions in the use of the other nine insecticides. Average application rates for these insecticides have declined from 0.37 lb/acre in 1995 to 0.12 lb/acre in 2000. USDA pesticide use data show that by 2000, growers in these six states used approximately 3.1 million pounds less insecticide than in 1995, accounting for 16% of all insecticide use in those states in 1995. The number of insecticide applications[1] has also declined, by 16.6 million applications in 2000, or 25% of the total number of insecticide applications in those states in 1995 (*15*). Table 2 shows reductions in insecticide use from 1995 and 2000, adjusted for acreage changes. Alabama growers in particular have achieved great reductions in insecticide use compared to 1995, making between 0 and 2 insecticide applications for bollworm/budworm since 1995.

Sweet Corn

Bt sweet corn varieties were commercialized in 1998. However, adoption has been very low thus far, due largely to fresh corn marketers' unwillingness to purchase Bt varieties. We have projected benefits of Bt sweet corn for Florida fresh sweet corn growers if they were to adopt the technology. Bt sweet corn

[1] *An application is the number of active ingredients applied per acre times the number of repeat applications, and differs from the number of trips over the field, as one trip across the field to apply two active ingredients is treated as two applications, as is two treatments each containing a single ingredient.*

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Table 1. Reductions in Cotton Bollworm/Tobacco Budworm/Pink Bollworm Insecticide Use After Introduction of Bt Varieties (AR, AZ, CA, LA, MS, TX)

Insecticide	*lbs (1000)*	*applications (1000)*
Amitraz	83	449
Cyfluthrin	13	2554
Cypermethrin	96	2349
Deltamethrin	-30	-243
Esfenvalerate	46	1247
Lambdacyhalothrin	109	3384
Methomyl	439	1569
Profenofos	1428	2650
Spinosad	-86	-1256
Thiodicarb	941	2834
Tralomethrin	28	1263
Zeta-cypermethrin	-9	-156
Total	3058	16643

SOURCE: Calculations based on USDA NASS Chemical Usage Survey Data (*17*)

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varieties with the cryIA(b) gene exhibit resistance to a range of insect pests similar to that of Bt field corn, though the prevalence and importance of these pests in sweet corn production differs from that of field corn. The primary target pest of Bt sweet corn in Florida fresh sweet corn production is the fall armyworm. The greatest impact of Bt sweet corn varieties is anticipated to be a reduction in insecticide use.

Florida is the number one fresh sweet corn growing state, accounting for approximately 25% of total U.S. production and value (*19*). Fresh sweet corn production in Florida is dependent on intensive insect management due to a high potential for insect damage, a low market tolerance for ear damage and high crop value. Indeed, economic sweet corn production in Florida was made possible largely through the use of insecticides (*20*). On average, Florida sweet corn acreage is treated 12 times with insecticides during the growing season (*21*).

Bt sweet corn varieties exhibit a high level of resistance to fall armyworm and other lepidopteran pests of Florida sweet corn, even under high infestation levels (*22, 23*). In field trials comparing Bt and conventional sweet corn conducted at the University of Florida, yields from the Bt plots were nearly 900 lbs higher, insect control costs were decreased by $70/acre, and the number of insecticide treatments was reduced dramatically (*24*). Bt sweet corn does not provide control of non-lepidopteran insect pests such as corn silk fly, which is another economic pest in Florida sweet corn producing areas. Sweet corn growers are not expected to be able to eliminate insecticide use altogether through the use of Bt sweet corn. We project that Bt sweet corn would be suitable for adoption on approximately 80% of the fresh sweet corn acreage in Florida where corn silk fly is not the primary pest. Where adopted, growers are expected to eliminate 10 of 12 insecticide sprays, or over 100,000 lbs. of insecticides across the state. Insect control costs are estimated to be reduced by $1.3 million per year, including the $77/acre technology fee. Production would be increased by approximately 22 million lbs/year, valued at $3.9 million (*20*).

Potatoes

Three types of Bt potatoes have been developed and commercialized: NewLeaf; NewLeafPlus with resistance to the potato leafroll virus; and NewLeafY with additional resistance to potato virus Y. The first type was introduced in 1996, the other two in 1999. All three Bt potatoes were engineered to produce the Bt Cry3(a) protein. NewLeafPlus and NewLeafY provide additional protection against virus diseases through the expression of viral coat proteins. Combined adoption of all three types of Bt potatoes has been

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limited, peaking at 4% of U.S. potato acreage in 1999 (*9*). The low adoption is attributed to marketing concerns, as processors responded to the refusal of fast food chains and other buyers to accept genetically modified potato products (*25*).

The target pest of all Bt potatoes is the Colorado potato beetle (CPB). Virus resistant varieties protect against diseases spread by aphids. We project the benefits of NewLeafPlus potatoes in the northwestern states of Idaho, Washington and Oregon, which together account for over half of U.S. potato production, and where growers face both CPB and potato leafroll virus. The major anticipated impact of the adoption of NewLeafPlus varieties in those three states is reduced pesticide use.

CPB is the primary defoliating insect pest of potato in the US. The CPB feeds on potato foliage, leading to reduced photosynthesis and yield. The green peach aphid is the primary insect pest for northwest potato growers due to their ability to transmit viruses such as the potato leaf roll virus (PLRV) that infect underground tubers (*26*). Potato leaf roll virus causes net necrosis in tubers, which lowers the marketable value of the crop. Potato growers routinely use insecticides to control CPB and aphids. Typically, 90% of potato acreage in Idaho, Washington and Oregon is treated with insecticides, accounting for 2 million pounds of insecticide use annually (*17*). Approximately 2/3 of these insecticides are targeted at CPB and green peach aphids. Control of CPB is particularly difficult, as it has developed resistance to a broad range of insecticides (*27*).

Control of CPB populations is greater than on nontransformed potato plants treated with insecticides, and protection against defoliation is season long (*7, 28*). NewLeafPlus potatoes exhibit high resistance to the potato leaf roll virus both in terms of visible symptoms and virus incidence (*29*).

Potato growers who adopt NewLeafPlus potatoes are expected to be able to eliminate the use of insecticides for CPB and aphid control. In addition, it is estimated that half of the use of another insecticide for mite control would be made unnecessary, due to the conservation of natural mite predators following the cessation of sprays for the CPB and green peach aphid (*30, 31*). The estimated reduction in insecticide use is approximately 1.5 million lbs/year, with an associated cost savings of $27 million/year, including the technology fee of $46/acre. Further, the reduced incidence of PLRV is expected to increase yields by 1680 lbs per acre, or $52 million per year total (*20*).

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Field Corn for Rootworm

Two new Bt field corn varieties have been developed which produce Bt proteins toxic to corn rootworm species. One variety, developed by Monsanto, produces the Cry3Bb protein at a low dose level. An application for its registration was submitted to EPA in March 2001 (*32*). The other variety, developed by Dow AgroSciences, Pioneer Hi-Bred, and Mycogen Seeds, produces a Bt protein at a high dose level and is currently being field tested (*32, 33*). The protein produced in the high dose variety has not yet been categorized as a Cry protein but is referred to as the PS-149-B1 protein. The primary impact of the introduction of Bt field corn varieties with protection against rootworm is expected to be a large reduction in insecticide use.

Corn rootworms are the most serious insect pests in field corn. There are four species of corn rootworm found in the U.S.: northern, western, southern and Mexican. Rootworms causing significant damage in the eastern and western Corn Belt and some southern states are the northern and western corn rootworm. Southern corn rootworm occurs in most areas east of the Rocky Mountains, but is only a potential economic threat in southern states where it overwinters (*34*). The Mexican corn rootworm is found in south central states such as Texas, its range having expanded north from Mexico and Central America (*35,36*).

It has been estimated that rootworms cost U.S. corn growers $1 billion annually in control costs and crop losses (*11*). Corn growers in 22 states surveyed in 1992 reported that without treatment, yield loss caused by corn rootworm infestations ranged from 0% to 15%, but could be as high as 50% (*11*). It is expected that Bt field corn for rootworm would be most likely to be adopted on acreage planted to continuous corn and areas where rootworm had adapted to rotations, where rootworm infestations are more common.

Low dose Bt corn events were evaluated for protection against western and northern corn rootworm (*37,38*) In terms of level of root damage and consistency of protection, the low dose transgenic varieties performed equally well or better than the soil insecticide treatments used for comparisons. The high dose Bt corn for rootworm control is estimated to provide complete protection from rootworm feeding, reducing economic losses to zero (*39*). Results indicated that rootworm protection provided by the high dose Bt event may be greater than the protection currently achieved with soil insecticide applications, preventing all losses from rootworm feeding.

The corn rootworm insecticide market is the largest pesticide market in the U.S., accounting for $22 million in annual sales, 15 million acres treated and 12

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million lbs of active ingredient (*11*). It is expected that the introduction of Bt field corn for rootworm control could replace a substantial portion of this insecticide use.

Soybeans

Bt soybean varieties are under development. Researchers at the University of Georgia have transformed soybean with the Cry1Ab and CryIAc Bt genes for protection against velvetbean caterpillar and lesser cornstalk borer (*40, 41*). In addition to the transgene-induced insect resistance, natural lepidopteran-resistance traits from Japanese soybean varieties were bred into the Bt soybean for broad-spectrum protection against corn earworm and soybean looper (*42*). Bt soybean is expected to have potential value in southern U.S. soybean growing areas, where insect pests are more problematic. Impacts are expected to include increased yields and reduced pesticide use.

In southern soybean production, the most damaging defoliating insects are velvetbean caterpillar and soybean looper (*43*). Other foliage feeders of economic significance include lesser cornstalk borer and corn earworm. Losses from velvetbean caterpillar alone in Georgia soybean may be as high as $2 million in combined damage and cost of control (*44*).

If commercialized, southern soybean growers, who spend an estimated $100 million per year on insecticide use, are expected to benefit most through insecticide use reduction. It is estimated that soybean growers in the southern US could reduce insecticide use by 295,000 lbs/year, or 67% of total insecticide use in soybeans (*20*).

Peanuts

Bt peanut lines have also been developed (*45*), expressing a CryIA(c) gene with efficacy against lesser cornstalk borer (LCB). Georgia is the number one state in peanut acreage, production and value, accounting for 40% of US peanut production. Georgia peanut growers face insect populations and associated fungal contamination of the crop which could be reduced with the adoption of Bt peanut varieties.

Larval LCB cause serious economic damage by feeding on parts of the plant at or just below the soil surface (*46*). In addition to direct damage, LCB feeding results in scars and wounds that facilitate infestations of soilborne plant

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pathogens such as Aspergillus fungi (*47*). Aspergillus fungi are of major concern to the peanut industry because they produce aflatoxins that are detrimental to human and animal health (*48*). Growth of Aspergillus fungi is especially favored in drought conditions. Peanut lots that are contaminated with Aspergillus are downgraded at a significant loss to the grower. An economic analysis, based on the non-drought years 1993–1996, estimated the average net cost to Georgia growers of downgrading due to Aspergillus contamination to be $1.7 million (*49*). Insecticides are used by peanut growers to control LCB to reduce aflatoxin levels in the crop.

Experimental Bt peanut lines demonstrated high control of LCB and moderate to high control of two other peanut lepidopteran pests (*50, 51*). It is expected that this level of efficacy would translate into reductions in aflatoxin levels. Bt peanuts may not solve aflatoxins problem in peanuts. However, it is estimated that LCB control could reduce fungal contamination by at least 50% and could reduce the current losses by $0.9 million per year (*20*). A reduction in chlorpyrifos-methyl use, the only insecticide currently available for the control of LCB, could result in a savings of $0.6 million dollars for Georgia growers alone.

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Conclusion

The pests that are controlled by genetically engineered Bt crops have been long-term problems for US growers. These pests have also been the subjects of large, publicly funded research programs. Genetically engineered insect-resistant crops are often times less expensive and more effective than conventional pest control methods. Impact assessments presented in this paper are based on preliminary studies or short-term field research. Environmental and economic conditions vary over time. Therefore, more accurate assessments may be made after long-term use of genetically engineered crop varieties. Nonetheless, at this point in time, it appears that Bt crops offer significant benefits to growers in terms of increased yields, decreased production costs, and reduced pesticide use. Corn and cotton growers continue to enjoy the benefits of Bt crops, while growers of potatoes, sweet corn, soybeans and peanuts would likely derive substantial benefits if marketing issues were resolved, allowing for the commercialization of these Bt crops.

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Chapter 4

Applications of Biotechnology for Improving the Healthfulness and Utility of Cereals

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The biotechnology approach allows geneticists and plant breeders to add improvements and value to cereals in ways that have not been easy or even possible by traditional breeding. Genetic transformation methods facilitate the introduction of one or a few genes from natural and man-made sources. These genes are chosen and designed to extend the food and non-food uses of cereals. This chapter highlights some applications of biotechnology for improving attributes of rice, wheat, maize and barley, the most important cereal crops for human consumption and utilization.

Cereal transformation

In 1988, rice was the first cereal plant to be transformed (*1, 2*), and this achievement was soon followed by similar successes in maize (*3, 4*), wheat (*5*) and barley (*6*). The first experiments with rice used electroporation to introduce DNA into protoplasts made from cells that were capable of regenerating into whole fertile plants (*1, 2*). Later experiments in the cereals utilized embryogenic callus cells derived from the scutelli of immature embryos as sources of such

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totipotent cells (*3-6*). The methods most commonly used now to deliver DNA to cells are biolistics (particle bombardment or the "gene gun") and *Agrobacterium tumefasciens*. Less commonly, DNA is introduced by pollen tubes, vortexing with fibers, injection or electroporation (*7*). Introduction of DNA by *Agrobacterium* has the advantage that insertion sites usually contain a single or few copies (*8, 9*). In contrast, particle bombardment more typically introduces multiple copies of the incoming DNA, often interspersed with host genomic DNA (*10-13*). Detailed histories and descriptions of transformation methodologies are well described in several recent reviews (*14-17*).

A typical transformation experiment includes at least two separate genes. These genes can be isolated from natural sources and/or they can be synthesized and assembled *in vitro*. The main focus of the experiment is on the "gene of interest", DNA that is expected or known to control some aspect of plant development or utilization. The second type of gene introduced in transformation experiments is a "marker gene". Its sole purpose is to facilitate the identification of transformed cells and plants. Marker genes include herbicide and antibiotic resistance genes. Transformants containing these types of marker genes are selected by including appropriate chemicals in culture media and/or applying the chemicals to plants. Marker genes can also encode protein products that are readily detected by visualization, such as the green fluorescence protein from *Aequorea victoria* (*18, 19*) or β-glucuronidase from *E. coli (20)*. Because the efficiency of stable transformation is typically only a few percent (that is, only a few of a hundred starting explants stably incorporate the incoming DNA into their chromosomes), marker genes are necessary for identification of transformants. However, once transformants are obtained, the marker gene is no longer needed and is, indeed, undesirable in plants that are intended for commercial release (see below).

In the earliest transformation experiments, most of the genes of interest were of agronomic value, chiefly encoding resistances to herbicides, insects and viruses. The transgenic crop plants produced in this first wave are widely perceived to chiefly benefit farmers and agribusinesses. However, they offer indirect benefits to consumers as well by shifting use toward more specific types of pesticides and less toxic types of herbicides (*21, 22*). A striking example of an indirect consumer benefit of a crop engineered for agronomic improvements is Bt corn, which was designed to be resistant to the European corn borer by expression of genes encoding a natural insecticidal protein from *Bacillus thuringiensis*. In addition to protecting the corn from insect damage, the presence of this protein also partially protects the plants from some types of fungi, including those that produce mycotoxins (*23, 24*). Reduction of these health hazards is a side effect of decreasing insect damage: the lower number of insect wounds provides fewer opportunities for fungal invasion.

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A second wave of transgenic cereals is now under development with traits that target consumer benefits more directly, whether by improving food healthfulness or cereal utility for food applications. A third wave promises to extend the utility of cereals beyond their traditional roles. These second and third generations of biotechnology applications in cereals are the subject of the next three sections.

Targeted Traits for Healthfulness

Several lines of biotechnology research aim to improve the nutritional properties of cereals. Of special interest and importance in this regard are experiments with rice, which is the chief food of half of the world population (*25*). To improve the nutritional content of rice, Potrykus and his colleagues at the Swiss Federal Institute of Technology-ETH in Zurich introduced a set of three genes that together give rice endosperm the capacity to accumulate β-carotene, the immediate precursor of vitamin A (*26*). A lack of this vitamin is implicated in blindness and premature mortality for millions of children who depend on rice as the major caloric component of their diets (*27, 28*). The same group of researchers has also added genes to rice to raise the iron content of the grain (*29*). Iron deficiency is the leading nutritional disorder in the world today, affecting as much as 30% of the global population (http://www.who.int/nut/). In Lucca et al.'s experiments, addition of a bean gene encoding ferritin increased the iron content of rice grains, doubling it in plants with the highest transgene expression (*29*). In contrast, introduction of a soybean ferritin gene did not increase the iron content of rice or wheat grains, even though increases in vegetative tissues were noted (*30*). Just raising iron levels is not sufficient to enhance the nutritional value of grain. Equally important is to package the iron in a bioavailable form that is readily absorbed by the human digestive tract (*31*). Toward this end, Lucca et al. added genes for a thermotolerant fungal phytase and a rice metallothionein-like protein rich in the amino acid cysteine (*29*). These genes had little direct effect on iron levels; their effects on iron absorption have yet to be determined. Other potential targets for nutritional improvement of grain vitamin and mineral content are discussed by DellaPenna (*32*).

Another target for nutritional improvement of cereal grains is balanced amino acid content. Cereal grains are notably deficient in the essential amino acids, lysine and threonine, and these deficiencies limit their utilization as protein sources by monogastric mammals. To make cereals closer to ideal sources of complete protein, genes encoding feedback-insensitive versions of an enzyme central to lysine biosynthesis were introduced into maize (*33*) and rice

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(*34*). This enzyme, dihydrodipicolinate synthase, is normally inhibited by free lysine. Expression of feedback-insensitive versions in rice resulted in increased accumulation of lysine in seeds and a more balanced amino acid composition. In maize, it was found that free lysine could not be increased in endosperm tissue because it triggered its own catabolism. However, overall grain lysine content was increased by expressing the enzyme in embryo rather than endosperm tissue (*33*). Experiments with similar goals are underway to raise the lysine content of wheat grains (Anderson, Galili, Gitt, Blechl, unpublished).

Targeted Traits for Utility

While rice grains are mainly consumed directly after cooking, wheat is milled into flour before being processed into a wide variety of edible products. One of the main determinants of the food uses of wheat is the strength of the doughs prepared by mixing flours with water. Dough strength is largely dependent on the seed storage proteins in wheat. A class of proteins called the high-molecular-weight (HMW-) glutenins has shown the best correlation with bread dough strength in genetic studies (reviewed in *35*). To better define the relationship of these proteins to dough functionality, our lab has introduced genes for natural and variant HMW-glutenins into a wheat cultivar that already contains genes encoding HMW-glutenin subunits Ax2*, Dx5, Bx7, By9 and Dy10 (*36*, Figure 1A). Using genetic transformation, additional copies of the native genes for Dx5 and Dy10 under control of their own promoters were introduced, resulting in increases in the levels of these proteins in endosperm. The transformant in lane T of Figure 1A contains 55% more Dx5 and 70% more Dy10 than its non-transformed parent (C), as determined by scanning densitometry of the gel.

In order to measure the effect of the addition of Dx5 and Dy10 on functionality, flour was prepared from seeds of plants homozygous for the transgenes. Two grams of this flour were mixed with water in a small-scale mixograph, an instrument that measures resistance as it mixes the dough. Dough development of the non-transformed control is shown in the upper panel as a trace of resistance vs. time (Figure 1B). After a brief hydration phase, continued work input from the rotating pins of the mixer increases resistance to a peak, at which time the dough is optimally mixed. Continued work input results in over-mixing and a decline in resistance. Measures of dough strength include the time and work input (area under the curve) to peak resistance. Measures of dough stability include the slope and the thickness of the trace after the peak. The non-transformed control flour has a mixing curve typical of moderately strong bread doughs, but with low tolerance for over-mixing, showing a steep decline in both

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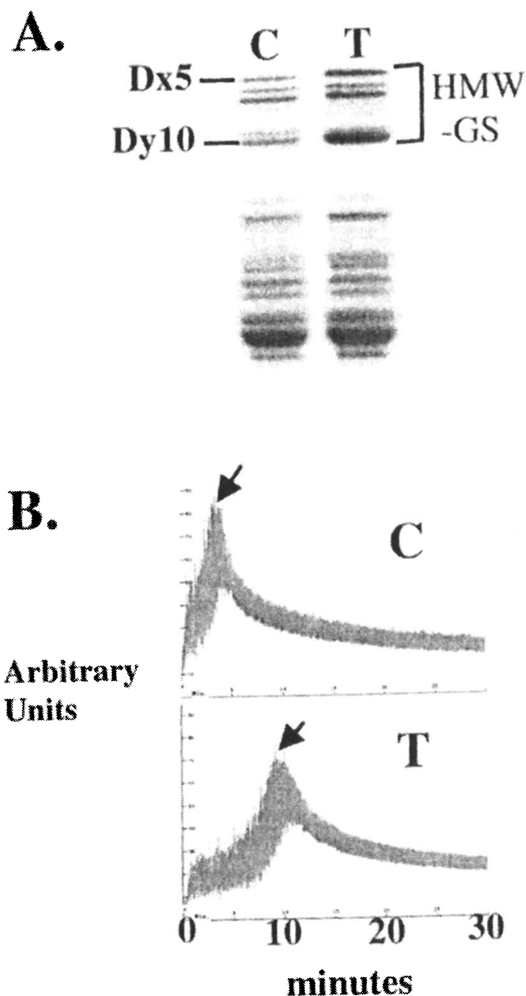

Figure 1. **A**. SDS-PAGE of seed extracts from wheat (T) transformed with genes encoding HMW-glutenin subunits Dx5 and Dy10 (dashes) or non-transformed control wheat (C). The location of the HMW-glutenin subunits (HMW-GS) in the gel is indicated by the bracket. **B**. Mixograms of 2 grams of flour milled from the plants in **A**. Resistance in arbitrary units is plotted against time of mixing. Arrows point to peak resistances.

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the height and thickness of the trace after the peak. The transformant with increased levels of Dx5 and Dy10 exhibits significant improvements in both mixing strength and tolerance (lower panel, Figure 1B). The optimal dough takes longer to develop with a thicker resistance trace. Resistance after mixing is more stable than the control, indicating a greater tolerance to over-mixing. These results are similar to those obtained by others using genes for x-type subunits transformed into other wheats (*37-39*).

Another target for genetic modification is the starch composition of grains, which constitutes most of their mass, comprising for example, 70% of wheat flour. Maize starch is widely used in food and non-food products because of the availability of a number of genetic variants, such as waxy and amylose extender, that produce starches with a variety of pasting and gelling properties (*40*). Relative to maize, wheat has relatively few types of starch. This is because bread wheat is a hexaploid plant so that the loss-of-function mutants that produce the variant starches of diploid plants such as maize are difficult to identify in wheat. Genetic transformation will allow the generation of new types of starch in wheat and other cereals (*41*). Modifications that are presently performed chemically on purified starches could be done *in vivo* by enzymes whose genes are introduced by transformation (*40*). Another approach would be to suppress the activity of specific genes that encode enzymes for starch branching, leading to different starch structures, expected to have different properties. For example, introduction of more branches or a decrease in amylose content is expected to slow retrogradation of wheat starch and could extend the shelf life of wheat products (*41*). Thanks to experiments in dicot plants that serve as model systems, an understanding of post-transcriptional transgene-mediated suppression has emerged that will allow experimenters to design constructs that reduce the expression of the targeted genes in predictable ways (*42, 43*). Alternatively, antisense constructs can be used to decrease gene expression of starch branching or biosynthetic enzymes (*44*).

The chief uses for barley grain are as feed and in brewing for beer. Targets for genetic engineering have mainly focused on improving the digestibility of grain components, particularly of carbohydrates during the malting process. Toward these goals, Jensen et al. (*45*) introduced a gene for a thermostable glucanase into barley and found increased digestion of cell walls at the higher temperatures typical of wort. Kihara et al. (*46*) introduced a thermostable ß-amylase into barley. These introductions are expected to improve the efficiency of the malting process.

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Future Challenges

The next wave of potential applications for cereal biotechnology will be to use cereal plants to make valuable non-food proteins in their seeds. In this regard, experiments in maize lead the way. Three proteins useful in scientific research, avidin, aprotinin and ß-glucuronidase have been produced on an industrial scale and purified from maize seeds (*47-49*). The highest expressing transformant contained 2% of its soluble protein as avidin. The proteins were stable in stored dry grain (*47*). The successful industrial-scale production and purification of biologically active proteins from grains open up many possibilities for extending the use of cereal crops beyond their traditional roles.

One such potential application is production of therapeutic proteins such as antibodies and vaccines in cereal grains. Although most of the work in this area so far has been done in the Solanaceous crops of potato, tomato and tobacco (*50*), some progress has been made in cereal grains. Two antibodies with potential use in fighting human cancer have been produced in wheat and rice (*51*). Antigens that could be purified and used for oral immunization have been produced in maize (*52*). Two other therapeutic proteins have been successfully produced: in maize, a trypsin inhibitor useful after transplantation surgery and, in rice, human α-1-antitrypsin, a protein potentially useful in treating liver diseases, cystic fibrosis and hemorrhage (*53*).

The progress made so far in genetic engineering of cereals has entailed the mastery of a number of technical challenges, first in devising reliable transformation methods and then in achieving expression of heterologous genes and accumulation of their protein products in cereals. An additional challenge yet to be met will be obtaining consumer confidence in the biosafety of these crops. There are many aspects of transgenic plants that are of concern to varied sectors of the general public (see for example, *21, 54*). Some of these concerns can be addressed by improvements in the technology. One central concern for many consumers is the presence of extraneous DNA such as antibiotic resistance and marker genes and their protein products in transgenic plants and the foods derived from them. All plasmid DNAs have antibiotic resistance genes so that their presence in bacterial cells can be selected. A straightforward method for eliminating such genes from transgenic plants is to separate them physically from the plant expression portions of the plasmid before transformation. This can be done by digestion with appropriate restriction enzymes and gel electrophoresis. When Fu et al. (*55*) employed this method, they found an unexpected side benefit: inserts containing only the genes for plant expression had fewer copies and simpler structures than inserts in transformants generated using intact circular plasmids.

Marker genes cannot be removed from transgenic plants until after transformation because they are needed to identify transformants. Several strategies have emerged to remove marker genes from transformed plants; three

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are applicable to cereal plants. One comprises the use of *Agrobacterium*-based vectors in which the marker and genes of interest are each flanked by separate transfer-DNA left and right border regions (*56, 57*). These vectors will often introduce the two DNAs into separate sites in the genome, allowing the experimenter to identify progeny in later generations in which the gene of interest has been separated from the marker gene by the normal process of genetic segregation. A second strategy is to flank marker genes with sequences that are targets for site specific recombinases, for example, *lox* sites. Once such constructs are integrated into the genome, the experimenter can make a genetic cross between the transgenic plant with the lox sites and another transgenic plant expressing the appropriate recombinase, in this example, *cre*, which mediates excision and loss of whatever DNA is located between the two target sequences. This strategy can also be used to simplify and resolve complex insertion structures by flanking the gene of interest with the recombinase target sites (*58*). A third strategy combines tactics of the first two. In this case, the marker genes are flanked by the inverted repeat ends of transposable *Ds* elements (*59*). The experimenter can use genetic crosses to introduce the *Ac* transposase, which catalyzes excision and reinsertion of the *Ds*-flanked marker genes to new and, in 25% of the cases, unlinked locations in the genome. Segregation can then be employed to isolate the transgene of interest in germplasm that carries neither the transposase nor the marker gene. Re-insertion of the transgene via *Ds* transposition has the additional benefit of favoring single- and low-copy regions of the genome, where gene silencing is much less likely (*59*).

The transgenic cereal plants of the future will likely contain these and other refinements that will make them more acceptable to consumers. At the same time, these plants will deliver direct benefits to consumers either through improved nutrition or by packaging some types of medicinal proteins. The growing and processing of grain is one of our oldest technologies and genetic transformation promises to put it to new uses in the service of humankind.

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Chapter 5

Enabling Grain Distribution and Food Labeling Using Protein Immunoassays for Products of Agricultural Biotechnology

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Protein immunoassays have been developed that allow assessment of a load of grain in the field utilizing USDA sampling protocols and statistical techniques that estimate the probability of a load containing biotech grain with high levels of confidence. Such applications are being implemented commercially on a large scale for both Roundup Ready soybeans and StarLink Cry9c corn. ELISA methods have been developed that can determine the concentration of Roundup Ready protein in processed fractions of soybean including defatted flakes, protein isolate, protein concentrate and toasted meal, and Cry9c protein in corn flour and meal. The use of these methods within a system of identity preservation enables the distribution of grain and labeling of finished foodstuffs in a way that food processors are able to comply with food labeling laws.

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During the 1990's agricultural biotechnology companies introduced novel transgenic plants that contain small bits of new DNA which, in turn, code for the production of novel proteins that confer to the plant a unique characteristic, such as herbicide tolerance or insect protection. These novel bits of DNA and protein can be found in many different tissues of the plant, including seeds and grain and some of the processed foods prepared from them.

There has been an explosive acceptance by farmers of these new crops since their introduction and many different plant species have been transformed including crops traded as large agricultural commodities. In the year 2000, approximately 63% of the soybeans grown in the U.S, 64% of the cotton and 24% of the corn were ag biotech varieties, and it has been estimated that 60-70% of all food products in the U.S. contain biotech ingredients.

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Testing for Biotech

With the introduction of new crops derived using biotechnology there has been a need to have tests that can detect them in consignments of raw agricultural commodities and finished foodstuffs. There are essentially two reasons why people would be interested in testing for biotech ingredients in food - potential food safety issues and consumer choice. The biotech crops that are currently in major commercial production have been approved by regulatory agencies in countries throughout the world who have determined that they are not a food safety issue.

The second reason for testing for biotech crops involves the concept of consumer choice. There are many important socio-economic issues associated with these new crops including matters of international trade, intellectual property, environmental effects, biodiversity, sustainable agriculture, religious/ethical considerations, etc. Given the size and complexity of these issues it is perhaps not surprising that there is a lack of consensus regarding agricultural biotechnology, and so the concept has evolved that if experts and policy makers can't agree about these crops, then consumers should be given the right to make their own decision about whether they would like to purchase and consume foods containing biotech ingredients. As a result, a growing number of countries have enacted laws specifying that foods containing biotech ingredients be labeled as such, and labeling laws have created a need for testing.

There are two test methodologies used for the detection and/or quantification of biotech ingredients. Immunoassays are used almost exclusively to detect the novel proteins produced by biotech crops, and the novel DNA is detected using the Polymerase Chain Reaction (PCR). Immunoassays for biotech crops are available in two general forms, field-based, lateral flow strip tests used primarily for testing seed, leaf and grain, and laboratory-based ELISA

methods for testing processed ingredients. PCR methods are available for either qualitative screening applications or more quantitative analysis of specific transgenic events.

An important consideration when testing in support of labeling biotech foods is whether the biotech event that is being tested for is approved or unapproved. If an event is unapproved the testing method need only be suitable for detecting the presence of the biotech ingredient, i.e., it does not need to be quantitative because the mere presence of the event is unacceptable. An example of this is testing for StarLink Cry9c corn that was approved for use in animal feed but found its way into food distribution channels (*1*).

Testing for approved events requires that the test method be quantitative or be able to determine if the concentration of the biotech ingredient is above or below a specified threshold concentration with a stated degree of statistical confidence. An important component of the current laws governing the labeling of foods containing biotech ingredients is the establishment of threshold concentrations, over which a food needs to be labeled regarding biotech content. For example, in the EU labeling laws went into effect in April of 2000 mandating that foods containing greater than 1% genetically modified ingredients must be labeled (*2*). A similar law went into effect in Japan a year later specifying a 5% threshold (*3*).

Food labeling laws define labeling thresholds in terms of "percent GMO" (genetically-modified organism). To determine the concentration of biotech ingredient in a food, the analyst determines the concentration of biotech protein or DNA in the sample and then estimates the %GMO concentration employing a number of assumptions such as protein expression levels or gene copy number in biotech plants. It is important to realize that protein methods report %GMO as the ratio of the weight of GMO to non-GMO in a sample (weight %) while real-time, quantitative PCR expresses %GMO as the ratio of transgenic DNA sequences to host DNA (mole %). These two definitions of %GMO do not necessarily yield the same result, and this is an important consideration when trying to use both methodologies throughout a complex food production, distribution and regulatory system.

As a food progresses down the processing line towards final product, the money invested in the product is greater, the risk to the producer is higher and the analysis for biotech ingredients becomes more complex. For these reasons it is preferable to establish the identity of the biotech ingredient as a raw material before it enters into the food production process and then control the ingredient throughout the process rather than to test for unknown ingredients along the way or at the finished food stage. In this scenario, testing is still necessary to confirm the identity of the ingredient at various control points along the way but the testing is of a sample containing known, rather than unknown, ingredients and therefore is much less complicated.

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Threshold Testing at Local Grain Elevators

To comply with labeling laws it is not necessary to determine the exact concentration of a biotech ingredient, only to state with confidence that the food does or does not contain biotech ingredients above a specified threshold. This can be accomplished by establishing the identity of a biotech crop at the level of the farmer and then maintaining a chain of custody throughout the entire production process. The most important point of control in the entire system is at the initial sale of grain by the farmer to the local elevator. At this point it is critical to establish whether the load contains biotech grain before it becomes pooled with product streams designated to be low or 'non-GMO'.

A key element of this type of testing is that it must be done on site at the grain elevator and must be completed before the truck unloads its contents. During harvest trucks are typically lined up in a queue waiting to unload (Figure 1). Truckloads of commodities like soy and corn are normally subjected to routine testing, inspection and grading at the elevator for such things as moisture or oil content, size, dockage, etc. The system begrudgingly accommodates waiting for these procedures because they determine the price that the hauler will get for the load, but waiting for a test result prevents the hauler from bringing in

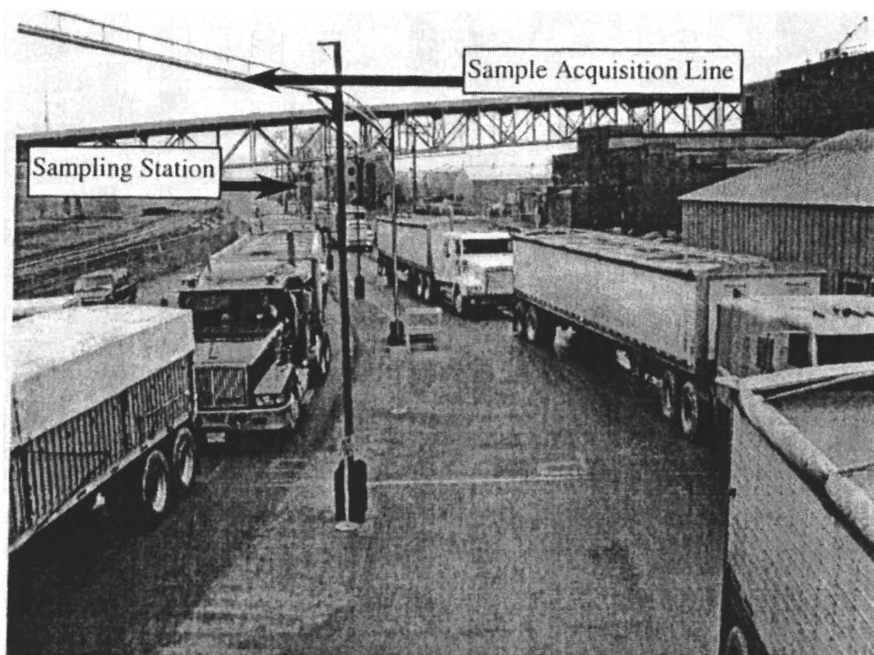

Figure 1. Trucks filled with corn waiting to unload at a local elevator.

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additional loads from the field. Therefore, cost effective tests for biotech commodities must fit within the normal testing regimen, i.e., utilize existing equipment, infrastructure and personnel and be executed during the time frame of the normal inspection process.

A testing methodology has been developed using rapid lateral flow test strips to detect the new protein found in a biotech crop in loads of harvested grain at local elevators. The contents of the truck are sampled according to procedures established by the United States Department of Agriculture's Grain Inspection, Packers and Stockyards Administration (GIPSA) using existing equipment and facilities. The performance of the test strips themselves is certified by GIPSA as part of their Rapid Test Performance Evaluation Program (*4*).

Figure 2 shows an example of a representative sample being taken from a truck using a vacuum probe. The representative sample moves through the sample acquisition line (Figure 3) to a location where normal inspection and grading procedures are performed and a portion of this sample is taken for analysis of biotech grain (Figure 4). A specified number of kernels or beans are ground in an ordinary kitchen blender or coffee grinder (Figure 5), shaken with a fixed volume of water and a small aliquot of the extract is incubated with the protein test strip for approximately 5 minutes. If the load contains greater than a specified concentration of biotech grain then the test reads positive, if the

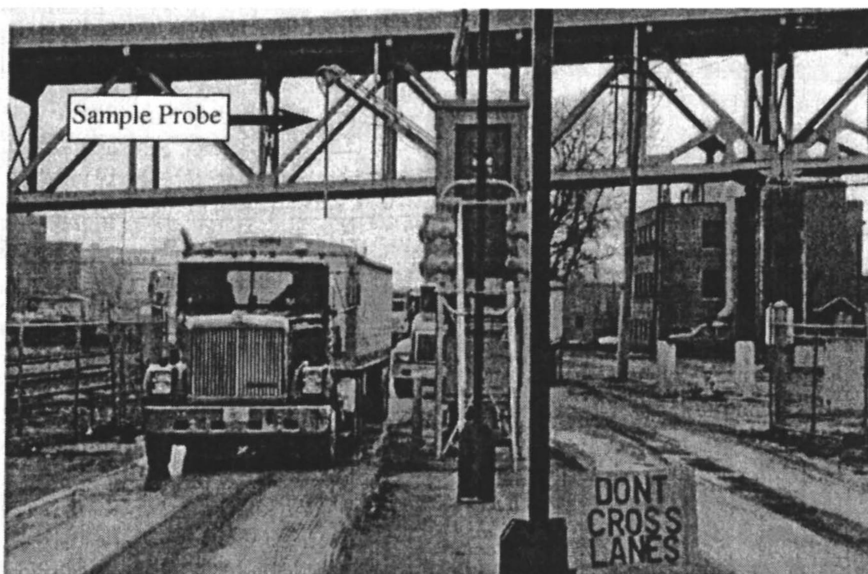

Figure 2. A representative sample of corn being taken from a truck with a vacuum probe.

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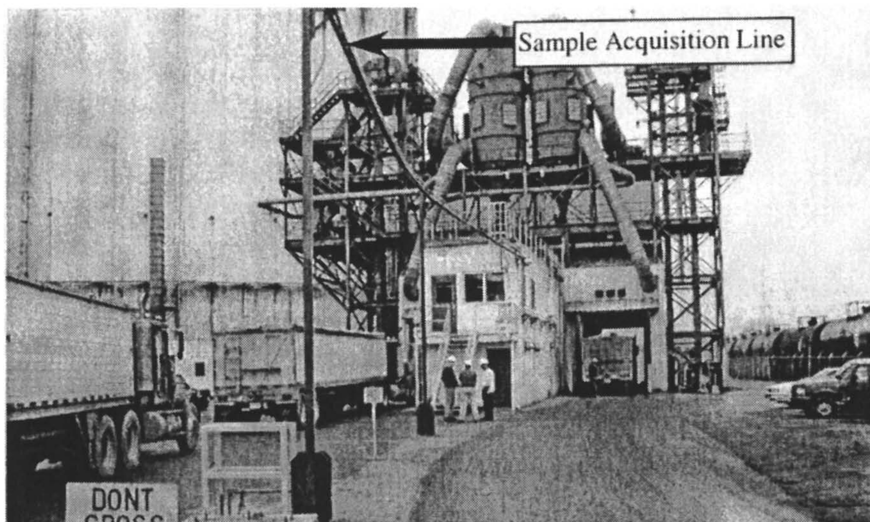

Figure 3. A representative sample of grain taken from a truck moves through a sample acquisition line to the sample testing area close to where the grain is unloaded from the vehicle.

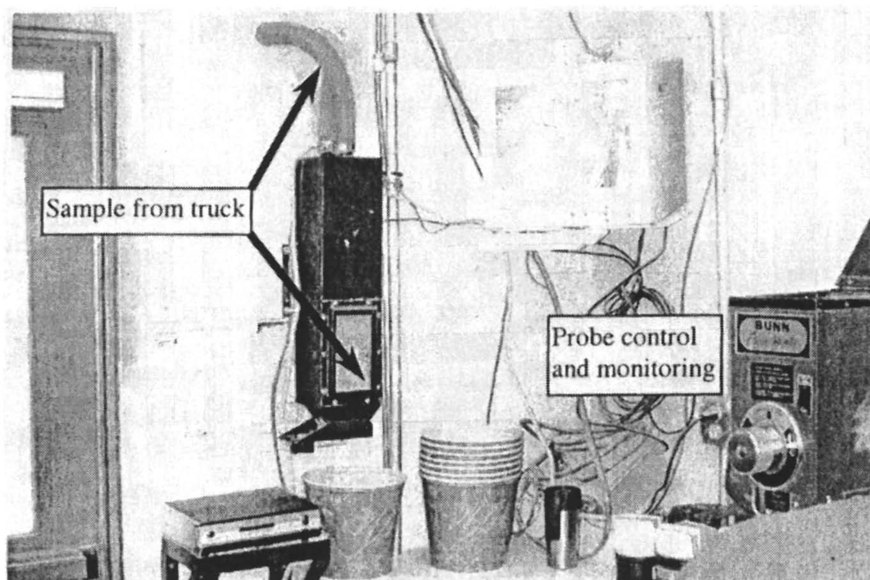

Figure 4. The representative sample from the truck is collected on site at a location where testing takes place.

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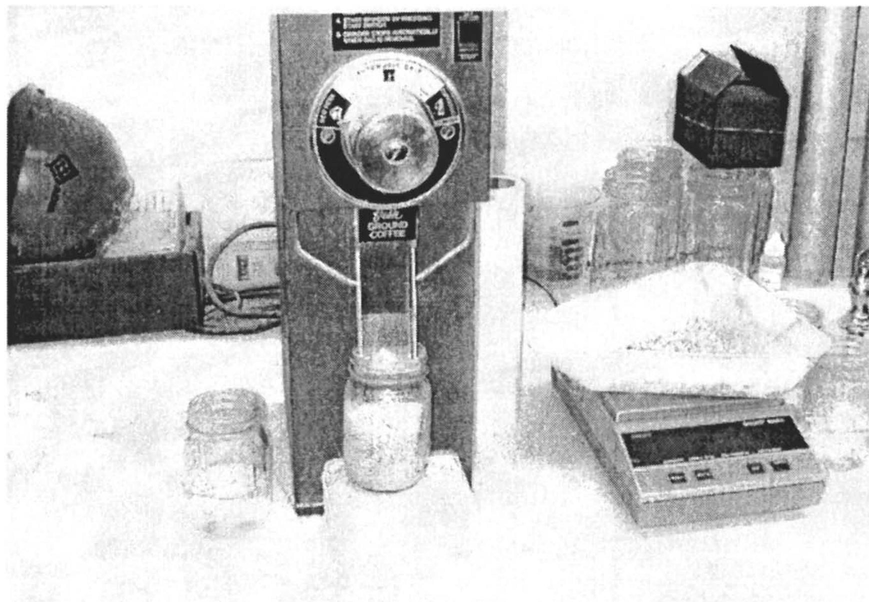

Figure 5. A fixed number of kernels are ground together, extracted with water and the extract is tested with a test strip.

concentration is below the critical threshold the test reads negative. Figure 6 shows the reactivity of a strip test for detection of *Bacillus thuringiensis* (Bt) Cry1Ab corn with various percentages of MON810 Bt corn mixed with 'non-GMO' corn.

The strip test methodology is based on a simple statistical procedure for estimating the probability of detecting one positive (biotech) kernel in a large number of negatives (*5*). Essentially the user defines the threshold concentration of biotech grain that they are interested in detecting (e.g., 1 or 5%) and the confidence they require for detecting that threshold (e.g., 95 or 99%), and then selects the number of samples to test in order to achieve the desired confidence. The number of kernels that can be ground together for analysis is determined by the absolute sensitivity of the strip test and is fixed by the test procedure in a way that the presence of a single biotech kernel in the sample will always give a positive response. The procedure is not quantitative in the sense that it does not report a discrete value, but just like a quantitative method, the result is reported in terms of statistical confidence in the outcome and thus the reliability of the result is the same as a quantitative method.

An important consideration when using protein tests in this manner is the natural variability of protein expression found in different varieties of biotech crops expressing the same protein, and even variability between kernels or beans

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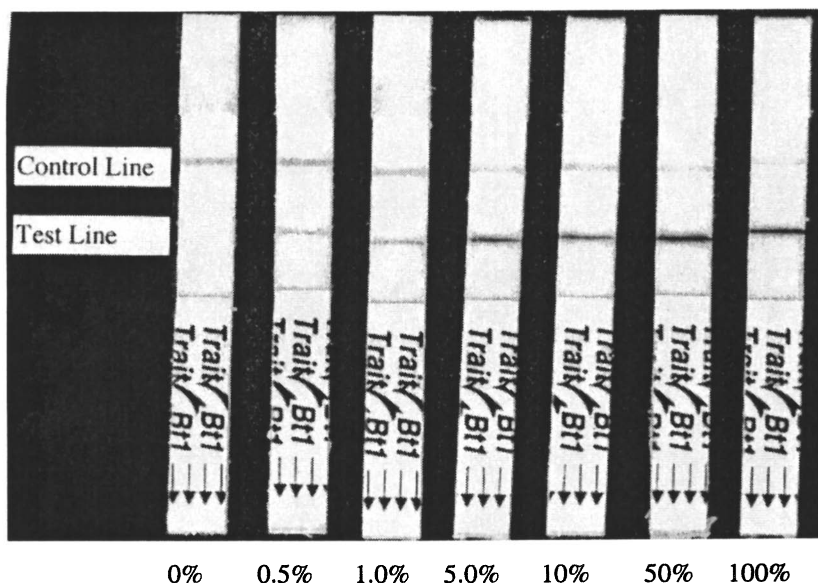

Figure 6. Reactivity of lateral flow test strips for Bacillus thuringiensis Cry1Ab protein with various percentages of MON810 corn.

within a single variety. The approach to managing this variability is to validate that the method is sensitive enough to detect the presence of a single kernel of the lowest expressing variant in commercial production. In practice, the developers of such tests evaluate as many different varieties as possible and the maximum number of kernels prescribed in the test procedure is designed to insure detection of the lowest expressing variants. Indeed, the GIPSA Rapid Test Performance Evaluation Program certifies test kit performance based on low-level protein expressing varieties.

Testing Processed Food Fractions

Testing of raw agricultural commodities is important because it characterizes the inputs to the food production process, but there are applications for testing that involve processed grain, such as flour and meal, highly processed intermediate food fractions and finished foods. The issues with testing processed food fractions are the same for both protein and DNA methods, i.e., quantification and validation. Figure 7 illustrates the performance of a Cry9c protein ELISA method that has been validated for quantifying StarLink corn in samples of corn flour and meal. Once the raw agricultural commodity, beans or kernels, are ground together and no longer exist as discrete units, it is not

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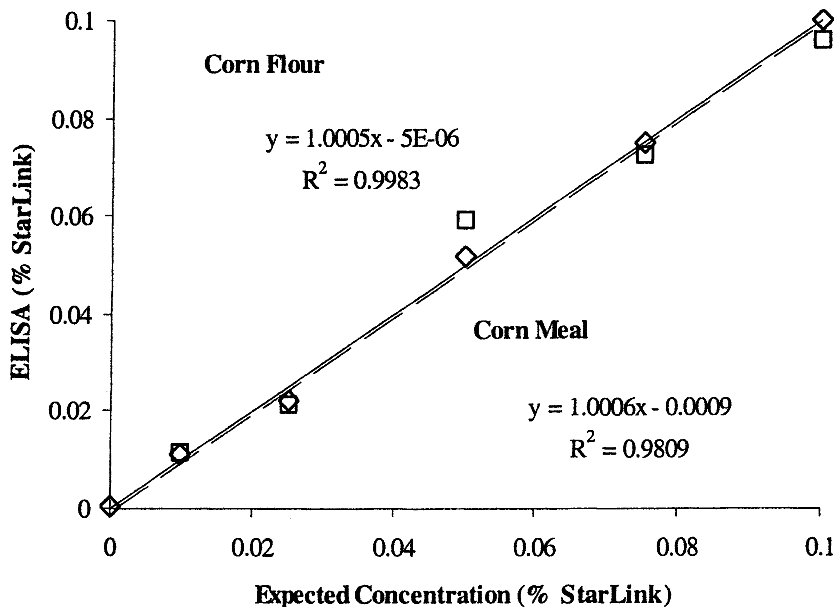

Figure 7. Quantitative protein ELISA methods have been developed for use with processed fractions of biotech grain such as StarLink Cry9c corn.

possible to use the statistical methodology used with the strip tests to determine the probability that the batch of material being tested is above or below the specified threshold concentration, therefore the test must be capable of assigning a discrete value for %GMO.

The issue with quantification is not simply whether a method can assign a %GMO value to a sample, but what the precision and confidence are around that value. Equally important is the accuracy of the test or how close the quantitative value is to the 'true' or 'correct' concentration in the sample. Factors that affect the precision of the final quantitative value include biological variability of the crop, the sample preparation procedure and the variability inherent within the analytical method. With respect to protein methods, biological variability (e.g., protein expression levels) can be relatively high and may significantly impact precision of the final quantitative value (*5*). DNA methods may be affected by variability from factors such as gene copy numbers within a crop species, the presence of 'stacked' genes in the sample (*6*), and the number of gene copies present in the aliquot of extracted DNA taken for PCR analysis (*7*).

The manner in which a sample is prepared may affect the capacity of a test to quantify both protein and DNA. Protein immunoassays that are capable of detecting novel biotech proteins in relatively unprocessed samples of flour and

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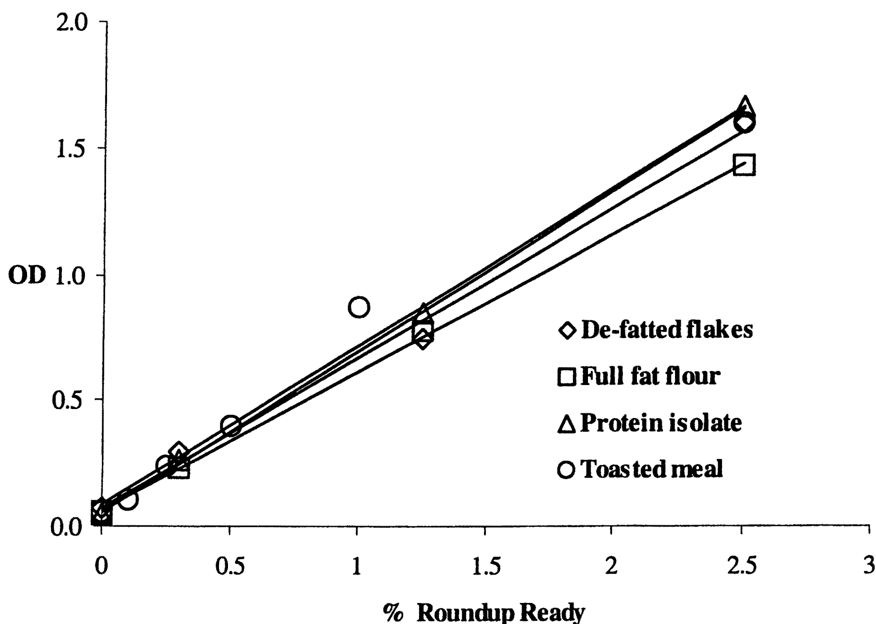

Figure 8. Protein ELISA method employing antibodies selected for specificity to processed and heat denatured fractions of soybeans.

meal may not detect these same proteins after they have been subjected to various degrees of processing (5). It is a common misconception that because some antibodies recognize target proteins based on the three-dimensional conformation of the protein, and that processing steps like heat denature such conformations, that antibody-based methods cannot be used on processed foods. This misconception exists despite ample evidence to the contrary (*8*). There are a number of strategies to overcoming conformational dependence of an immunoassay by developing antibodies that recognize the denatured protein, including immunizing animals with protein that has been deliberately denatured and peptide fragments representing continuous epitopes of the protein. Figures 8 and 9 show examples of protein immunoassays for CP4 EPSPS that have been validated with highly processed fractions of soybean.

While it is generally held that DNA is a relatively stable molecule and therefore not susceptible to differences in sample processing, it has been demonstrated that small differences in the procedure of preparing even relatively lightly processed ground soybean certified reference materials affected the capacity of PCR to quantify GMO (*9*), while a protein ELISA method was unaffected (*10*). In an ongoing effort to evaluate methods for measuring biotech

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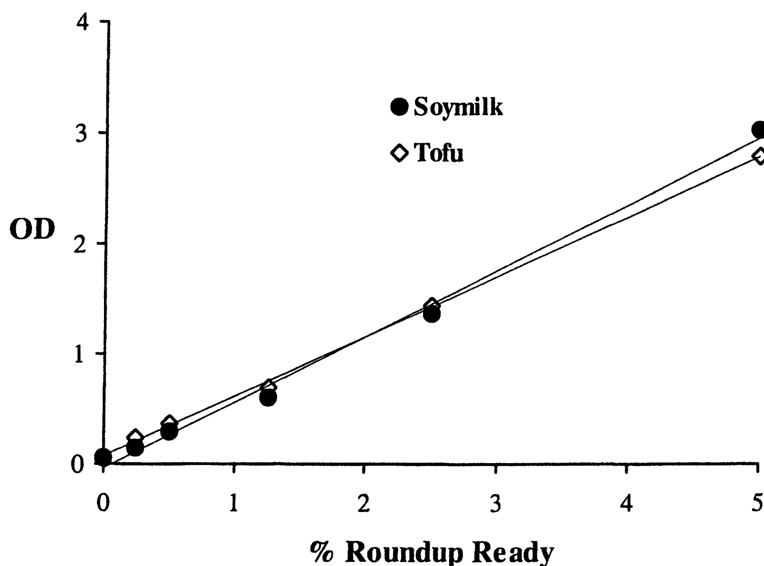

Figure 9. ELISA method for quantification of Roundup Ready in processed fractions of soybean.

soybean and corn in processed foods, the Working Party on Food Authenticity in the United Kingdom prepared samples of finished foodstuffs containing known concentrations of biotech ingredients and analyzed the samples by real-time quantitative PCR. To date, the results have shown that this technique can differentiate between foods made from "GM-free soya" and those containing much higher levels of biotech soy (i.e., qualitative analysis), but that when the concentration of biotech soy was approximately 1% GMO, "...accurate measurement may only be obtained by analysing the soya ingredient before processing" (*11*).

To prove that a test yields accurate results it is necessary to prepare the specific sample type from starting materials containing known concentrations of biotech ingredients according to the same procedure by which the sample is made. In this way the protein and DNA have been subjected to the same processing as the sample, and other substances co-extracted in the sample that may interfere with the performance of the test are the same. 'Spiking' purified protein or DNA into extracts of food samples provides information regarding interfering substances and matrix effects but does not control for the direct effects of sample processing on the target protein and DNA. Even relatively simple additions to a food substance can impact the performance of a method.

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For example, a method validated for corn flour may not yield the same results with a blended flour composed of corn, wheat, rye and barley (*9*). Ideally, it would be desirable to have highly characterized reference materials containing known concentrations of biotech ingredients prepared by all manner of different procedures to use as standards and calibrators for test methods (*12*). In reality this is not a practical solution.

Production of reference materials is difficult, costly, and to-date, only a handful of such reference materials have been produced. All of these reference materials are essentially ground grain rather than processed food fractions. Given the proprietary nature of food production processes, and the magnitude, cost and complexity of the task to make such reference materials it is likely that only limited numbers and types of references will become commercially available. Unless a method is validated with the sample it is intended to be used with the accuracy of the method cannot be determined and unless or until standardized reference materials become available the true accuracy of quantitative GMO tests remains an open question.

Testing Strategies for the Future

Given the considerable variabilities affecting the precision of quantitative GMO test methods and the uncertainties associated with accuracy, quantification of GMO in processed food fractions and finished foods remains a complex, costly and time consuming endeavor. At present, the primary motivation for testing is to determine whether a sample contains unapproved biotech ingredients or whether a sample contains approved ingredients above or below a threshold concentration to assess compliance with food labeling regulations. In the first case quantitative methods are not necessary, in the second, a discrete value is not necessary, only high confidence that the value is below the specified threshold.

It has often been observed that the United States favors protein testing while Europe prefers DNA testing. One of the reasons for this is the difference in rationale for testing. The United States is a producer of biotech crops and its' focus is on control and distribution. The objective of testing in this case is to manage business risk, and rapid, cost-effective protein tests are valued at a premium. Threshold testing methods of grain that yield very high statistical confidence in the outcome of the test, provide the reliability required to control biotech ingredients in the system.

Europe is primarily a consumer of biotech products and the objective of testing is to determine regulatory compliance. In this case, the cost and time of analysis are not the primary factors governing testing. The analysis is necessarily complex because a laboratory may test any food substance and attempt to determine the concentration of any and all potential biotech ingredients. The

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analyst has no prior information about the types and concentrations of biotech ingredients that may be in the sample, and has no reference materials with which to calibrate the method. Unfortunately, as long as matrix-matched reference materials are not available and methods have not been validated with the specific food substance to be tested, the reliability of the quantitative result is open to technical challenge.

Quantitative analysis of an unknown sample is complicated, time-consuming and costly, and for these reasons will be limited to applications where the stakes are high. An alternative to quantitative analysis is the implementation of identity preservation systems that establish the biotech status of the crop when it is harvested and then control the distribution and processing of the material in a way that the concentration is known at any given point along the way. It is still important to test at critical control points along the entire process to make sure that there hasn't been an inadvertent error along the way, but in this scenario, the objective of the analysis is simply to confirm the identity and concentration of a known sample. In this way the analysis is very much simplified and does not need to be quantitative. Rapid protein tests capable of establishing the biotech status of consignments of grain with high degrees of statistical confidence at the front end of the food production chain are an important component of a larger identity preservation system that enables food producers to label finished foods in compliance with food labeling laws.

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Chapter 6

Development of Polymerase Chain Reaction Methods to Detect Plant DNA in Animal Tissues

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To better understand reported detection of DNA fragments of high copy plant genes in animal tissues, two polymerase chain reaction (PCR) methods (conventional PCR followed by Southern blot and TaqMan real time PCR) were developed to detect fragments of the maize rubisco *rbcL* gene in beef liver, kidney, and spleen, and chicken spleen. When sample processing, DNA extraction, and PCR were performed under HEPA-filtered airflow, two TaqMan PCR methods, one being RT-PCR, did not detect the *rbcL* gene or its transcription. Only one of 33 samples was positive for the *rbcL* gene by conventional PCR under these controlled lab conditions. By comparison, the conventional PCR conducted under standard laboratory conditions showed substantial putative detection of the *rbcL* gene fragment in beef liver and spleen tissues, but extraction buffer-only controls showed similar results, thus indicating that these data were invalid. Therefore, a key conclusion is to avoid inadvertent DNA contamination that can confound data interpretation when using extremely sensitive PCR assays for highly abundant plant genes.

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Introduction

With the arrival of genetically modified food and feed products for human and animal consumption, questions have been raised regarding the digestive fate of transgenic DNA and protein. Although DNA, including transgenic DNA from genetically enhanced plants, is considered a safe and natural component of food according to the US FDA (1) and FAO/WHO (2), concerns have been raised over the detectability of transgenic DNA and protein in raw animal products.

As recently reviewed (3), numerous studies on the digestive fate of plant DNA have been conducted to clarify questions of whether DNA fragments can be found in the gastrointestinal tract, if there is any transfer of DNA into the body and possible incorporation of the ingested DNA into the animal genome.

It was shown in beef steers that over 50% of ingested plant DNA degrades in the first third of the ileum and approximately 80% is degraded after passage through the final ileum (4). In pigs, chloroplast DNA fragments could be found in all parts of the gastrointestinal tract up to 12 h after food intake (5). An experiment in which mice were fed daily with 50 μg of purified M13 circular or linearized phage DNA showed that approximately 0.1% of the original DNA could be detected in white blood cells as small fragments 2-8 h after feeding (6). In another study, no germline transmission, genomic incorporation or functional expression of a vector cassette of purified pEGFP-C1 plasmid (encoding Green Fluorescent Protein) was observed in mice fed high levels of this DNA construct either acutely or over eight generations (7).

The present study focuses on the question of whether it is possible to detect short DNA fragments in animal organ tissues. The selected gene was a high copy number plant specific gene (*rbcL*) encoded within plastids for the large subunit of ribulose-1,5-bisphosphate carboxylase (rubisco). To answer this question, two different polymerase chain reaction (PCR) methods were established and optimized in terms of sensitivity and specificity. One assay employed conventional PCR methods followed by a radioisotopic Southern blot analysis that allowed a limit of detection (LOD) of 10-100 copies of the *rbcL* gene which represents approximately $1/100^{th}$ of a cell genome equivalent. The other PCR assay, based on TaqMan real time detection and RT-PCR technology, reached a similar LOD.

Materials and Methods

Collection, Preparation and Homogenization of Animal Tissue Samples

Beef samples

Beef liver, kidney and spleen samples were obtained from a feed performance study that maximized the amount of maize in the diet (8).

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The beef steers were slaughtered after qualifying for the grade of USDA Choice (approximately 120 days after changing their diet) and organ and muscle tissue samples from 20 animals were shipped to the laboratory on dry ice. In the present study, DNA was extracted from 11 samples, each, of kidney, liver and spleen.

To reduce possible contamination with plant DNA during the slaughtering and sampling process, core samples were taken from the organs with cutting tools that were cleaned with bleach every time they contacted external surfaces. The resulting core samples were separated into subsamples weighing 2-8 g each. During all subsampling procedures, tissues were maintained on dry ice. After subsampling, all tissues were stored at –20°C.

Cell Lysis Buffer [CLB; 10 mM Tris-HCl, pH 8.0, 50 mM EDTA and 1% (w/v) SDS] was added to samples at a ratio of 4 ml CLB per 1 g tissue and homogenized with a blade homogenizer (Brinkmann Polytron PT3000). DNA was extracted from two 100-200 μl aliquots of the homogenate. As positive extraction controls, a pair of aliquots from one homogenate of tissue from a control animal were each spiked with 250 pg of maize genomic DNA. Two aliquots of CLB were taken through the entire DNA extraction process as negative controls.

Chicken samples

The chicken samples were obtained from a feed performance study that maximized the amount of maize grain in the diet (9). After 42 days of feeding, birds were randomly selected at the time of slaughter for tissue collection. Breast muscle and spleen samples from 20 chickens were collected and shipped to the laboratory on dry ice for subsampling and DNA extraction. Tissue subsampling methods were similar to those described for beef samples and tissues were maintained on dry ice during subsampling and subsamples were stored at –20°C.

DNA Extraction, quantification, and quality control for conventional PCR

Homogenates were treated with RNase (Roche) for 30 min, extracted with 25:24:1 phenol/chloroform/isoamyl alcohol (Roche) and subsequently centrifugated. The DNA was precipitated with an equal volume of 100% isopropanol (EM Science) and 1/3 volume 5 M ammonium acetate (Sigma). After an additional centrifugation and a washing step with 70% (v/v) ethanol (Aaper), the resulting pellets were air-dried and rehydrated in 1× TE Buffer (Sigma) overnight (~18 h) at room temperature or alternatively for 1 h at 65°C.

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The amount of DNA in each extract was quantified using the *fmax* Fluorescence Plate Reader (Molecular Devices) against a standard curve of several dilutions of DNA Molecular Weight Marker IX (Roche) and Hoechst dye (BisBenzimide H 33258, Sigma) according to manufacturer's instructions, and DNA extracts were adjusted to equal concentrations.

The following primers designed to amplify a 414-bp region of the endogenous preprolactin (*prl*) animal gene were used to analyze 100 ng of each DNA extract for potential PCR inhibitors. Calf thymus DNA (Sigma) was used as a positive PCR control and a reaction without template DNA was performed as a negative control.

- *prl* forward primer: 5'-CCA TGG ACA GCA AAG GTT CGT-3'
- *prl* reverse primer: 5'-TAT TCC AGT CTA AGT TTT CTC AA-3'

The resulting PCR products were analyzed on 2% (w/v) agarose gels with ethidium bromide (Sigma) staining and UV illumination for visualization.

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Extraction of genomic DNA and total RNA for TaqMan analysis

Genomic DNA was extracted from beef or chicken spleen by homogenizing tissue in 1× PBS. Qiagen Proteinase K (10 mg/ml) was added to the homogenate and the mixture was incubated at 60°C for 2 hours. Samples were diluted in 1/10 volume of 5M ammonium acetate (Sigma) and 3× volume of cold 100% ethanol (Aaper). DNA was mixed and spooled from a tube using a pipette tip. DNA was washed by swirling the tip in fresh 70% ethanol and air-dried. DNA was resuspended in 1× TE pH 7.5 (Sigma) by tapping the spooled DNA from the tip and heating the sample at 60°C for 30 min, followed by brief vortex mixing to complete resuspension. Lastly, 50 μg of DNA were subjected to the Qiagen MinElute PCR purification kit protocol, thereby purifying DNA from 70 bp - 4 kb (Qiagen).

Total RNA was extracted from beef or chicken spleen by homogenizing 250 mg of tissue in RNA-STAT (Tel-test Inc.), according to the manufacturer's protocol. Briefly, chloroform was added to each tissue homogenate (0.2 ml chloroform per ml RNA-STAT), each tube was shaken vigorously by hand for 15 s and incubated at room temperature for 2-3 min and centrifuged. The upper aqueous phase was carefully removed by aspiration and transferred to a clean tube on ice. An equal volume of phenol/chloroform/isoamyl alcohol (Ambion), pH 6, was added to the aspirated aqueous phase and contents were vortex mixed. Samples were re-precipitated by centrifugation. The aqueous phase was removed by aspiration and glycogen (Ambion) plus 500 μl isopropanol (JT Baker) were

added and each tube was vortex mixed. Samples were incubated at room temperature for 10-20 min, precipitated by centrifugation and the supernatant was decanted as waste. The RNA pellet was washed with chilled 75% (v/v) ethanol (Aaper), air dried and resuspended in 50 μl nuclease-free water (Ambion). RNA was treated with DNase using an RNase-Free DNase kit (Qiagen) following the manufacturer's instructions. The resulting product was extracted with 1:1 phenol/chloroform (Ambion) and precipitated with 5 M ammonium acetate (Sigma), glycogen and ice-cold 100% (v/v) ethanol. The RNA pellet was washed with chilled 75% (v/v) ethanol, air dried and resuspended in 50 μl nuclease-free water. DNA and RNA concentrations were determined using a spectrophotometer and sample quality was assessed with an Agilent Bioanalyzer 2100 or agarose gel electrophoresis.

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cDNA Synthesis

cDNA was synthesized using total RNA, random hexamers, oligo-dT and the Multiscribe Reverse Transcriptase kit (Applied Biosystems Inc.), according to manufacturer's instructions. The resulting RT+ and RT- products were assayed for quality and genomic DNA contamination, respectively, via single-plex TaqMan PCR using reagents specific for the 18S rRNA gene (Applied Biosystems Inc.) and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc.).

The conventional *rbcL* PCR assay

The primer pair chosen was compared to other known sequences to confirm its specificity to maize *rbcL* (Andreas Wurz, GeneScan, personal communication). The probe used for Southern blot analysis was sequenced for further confimation of the PCR product.

Aliquots (1 μg) of extracted animal DNA were tested for the presence of *rbcL* fragments. The positive PCR control was a reaction that included 10 pg of maize DNA, and the negative PCR control reaction lacked template DNA. To minimize carryover of amplicon, all PCR reactions included a nucleotide mix containing dUTP instead of dTTP (dNTP^{PLUS}, Roche). Uracil-DNA-glycosylase (UDG, Roche) was added to the PCR master mix and the reaction was pre-incubated for 20 min at 37°C to destroy any carryover amplicon. The UDG incubation cleaved uracil-containing products and prior to initiating PCR cycling, UDG was inactivated by incubation for 5 min at 95°C. The cycling conditions were 95°C for 30 s, 61°C for 30 s, 72°C for 45 s, and final extension after 35 cycles at 72°C for 5 min in an MJ Research PTC-225 Peltier thermal cycler. The PCR product with these primers is predicted to be 173 bp.

- *rbcL* forward primer: 5'-AGC TAA TCG TGT GGC TTT AGA AGC C-3'
- *rbcL* reverse primer: 5'-TGG TAT CCA TCG CTT TGA AAC CA-3'

The reactions were separated on 2% (w/v) agarose gels. DNA was made visible by staining the gels with ethidium bromide (Sigma) and UV light illumination. After visualization, the gels were treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) followed by neutralizing solution (0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl), and the DNA products were transferred to a Hybond-N nylon membrane (Amersham) with a TurboBlotter™ (Schleicher & Schuell) in 20× SSC buffer (Roche). DNA was crosslinked to the nylon membrane using a UV Stratalinker 1800 (Stratagene). DNA hybridization was performed with probes generated by a random priming method (RadPrime DNA Labeling System, Gibco Life Technologies) with incorporation of ^{32}P-dCTP (Amersham Pharmacia Biotech). Template DNA was purified PCR amplicon from the *rbcL* reaction. X-ray film (Kodak BioMax MS-2) was exposed to the membranes at room temperature for up to 50 min in the presence of one Kodak BioMax intensifying screen.

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The TaqMan *rbcL* PCR reagents

Two distinct, non-overlapping reagent sets that are specific for the *rbcL* gene were designed using Primer Express Software v1.5 (Applied Biosystems). Specificity of these designs was assessed via BLASTN 2.2.2 analysis. The sensitivity and efficiency of these primers (F1, R1, F3 and R3) and probes (P1 & P2) at amplifying a synthetic amplicon for *rbcL* (A1 & A3) while multiplexed with TaqMan reagents for human 18S (Applied Biosystems) was determined via TaqMan by standard curve analysis of known concentrations of amplicon.

- RbcL_1:
  - forward primer F1: 5'-GCC TGT GTA CAA GCT CGT AAC G-3'
  - reverse primer R1: 5'-CAC TCC ATT TGC AAG CTG CTT-3'
  - TaqMan probe P1: 5'-AGG GCG CGA TCT TGC TCG TGA A-3'
  - amplicon A1 size 69 bp: 5'-AGC CTG TGT ACA AGC TCG TAA CGA AGG GCG CGA TCT TGC TCG TGA AAA AGC AGC TTG CAA ATG GAG TGC-3'

- RbcL_2:
  - forward primer F3: 5'-GGA GGA TTC ACC GCA AAT ACT ACT-3'
  - reverse primer R3: 5'-TGC TCG GTG AAT GTG AAG AAG T-3'
  - TaqMan probe P2: 5'-TGT CTC ATT ATT GCC GCG ACA ACG G-3'

- amplicon A3 size 76 bp: 5'-AGG AGG ATT CAC CGC AAA TAC TAC TTT GTC TCA TTA TTG CCG CGA CAA CGG CCT ACT TCT TCA CAT TCA CCG AGC A-3'

- Human 18S:
  - forward primer 18S: 5'-GCC GCT AGA GGT GAA ATT CTT G-3'
  - reverse primer 18S: 5'-CAT TCT TGG CAA ATG CTT TCG-3'
  - TaqMan probe 18S: 5'-ACC GGC GCA AGA CGG ACC AGA-3'

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The TaqMan *rbcL* PCR assay

Beef and chicken genomic and cDNA samples were assayed for the presence of *rbcL* via multiplex TaqMan PCR. TaqMan probes for *rbcL* were labeled at the 5' end with the FAM reporter dye and at the 3' end with the quencher, TAMRA. 18S gene TaqMan probes were labeled at the 5' end with the VIC reporter dye and at the 3' end with TAMRA. A 50 µl PCR was performed using 10 µl of cDNA, 25 µl of TaqMan universal PCR master mix containing ROX as a passive reference (Applied Biosystems Inc.), 900 nM of each *rbcL* primer, 250 nM of *rbcL* probe, 100 nM of each 18S primer, and 200 nM of each 18S probe. Amplification and detection were performed with an ABI Prism 7700 sequence detection system under the following conditions: 2 min at 50°C to require optimal AmpErase uracil-*N*-glycosylase activity, 10 min at 95°C to activate AmpliTaq Gold DNA polymerase, and 40 cycles of 15 s at 95°C and 1 min at 60°C. During amplification, the ABI Prism sequence detector monitored real time PCR amplification by quantitatively analyzing fluorescence emissions. Signals from the reporter dyes (FAM & VIC) were measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well using the ABI Prism 7700 Sequence Detection System instrument (Applied Biosystems Inc.) according to the manufacturer's protocol for TaqMan assay PCR.

Results

Determining the LOD of the conventional *rbcL* PCR assay

In order to determine the sensitivity of this PCR assay and to test for nonspecific amplification of DNA from the three beef tissue types being studied (spleen, kidney and liver), PCR master mixes (i.e. a mix of all reagents necessary for PCR except the template DNA) containing DNA extracted from each of the different animal organ tissues were added to various concentrations of plant

DNA. As controls for potential contamination introduced by the animal matrices, a reaction was performed without plant DNA in the presence of each matrix. To determine potential influences of the animal DNA matrices on assay sensitivity, one PCR master mix contained 1× TE buffer instead of animal DNA.

Preliminary PCR results showed that amplification of an initial input of 50-100 fg of plant DNA (not in the presence of animal matrix or extraction buffer) produces a visible band on an ethidium bromide-stained agarose gel (data not shown). After subjecting the gel to Southern blot analysis, it was possible to detect 100, 10, 10 and 50 fg of maize genomic DNA in the presence of matrix from kidney, liver or spleen or with only extraction buffer (no DNA), respectively (Figure 1). The mass of DNA from a single haploid maize cell is approximately 2.4-3.7 pg (10). A single cell includes 1,000 to 10,000 copies of the plastid genome (11). Therefore, the ability to detect 10-100 fg of input plant DNA suggests that this assay is capable of detecting approximately 10-100 copies of the maize plastid *rbcL* gene. There are big differences in the copy number of plastids per cell among different plant tissue types and the number of genomic copies within plastids can also vary, therefore, it is not possible to precisely define an exact LOD value but instead only estimate an order of magnitude for the LOD of the *rbcL* PCR. Figure 1 also shows that no inadvertent plant DNA contamination occurred during PCR because the no-template control did not produce a band.

Analysis of beef tissue samples with the conventional *rbcL* PCR assay

DNA was extracted in duplicate from 11 samples each of beef kidney, spleen and liver. To an additional homogenate of each of these tissue types, 250 pg of maize DNA were added as a positive extraction control ("spike"). As a negative extraction control, the extraction procedure was performed using cell lysis buffer only.

Each set of samples (consisting of test materials, a spiked extraction control, and an extraction buffer control) was then extracted separately from other sample sets to avoid cross-contamination. After quantification and adjustment of the DNA concentration in each sample, a preliminary PCR with the extracted DNA was conducted to test for possible PCR inhibitors. For this experiment, primers for preprolactin (*prl*), a low-abundance endogenous animal gene, were used. All extracted DNA samples produced the expected *prl* amplicon indicating that all DNA samples were suitable for further PCR experimentation.

Table I shows the definition of accept/reject criteria along with the initial *rbcL* PCR/Southern blot analysis of 33 beef samples (11 DNA extracts each from spleen, kidney and liver). Six out of the 11 liver samples and 10 of 11 spleen samples were potentially positive for the *rbcL* gene fragment by the conventional PCR assay. None of the kidney samples appeared to be positive in

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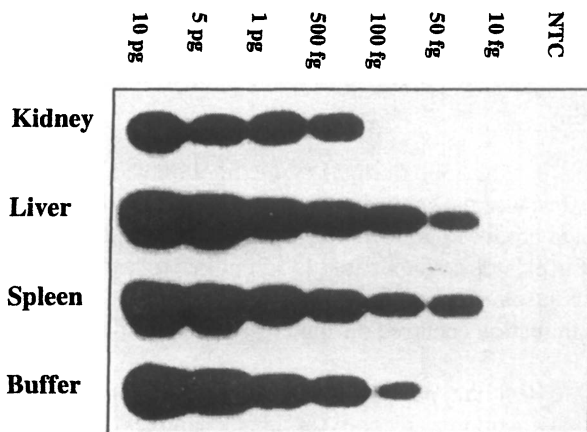

Figure 1: Conventional rbcL *PCR LOD experiment for maize DNA in the presence of beef organ matrix. The amount of input maize genomic DNA per reaction and the No Template Control (NTC) are shown across the top of the figure. Each reaction was spiked with either kidney, liver or spleen matrix, or extraction buffer alone.*

Table I. Conventional PCR/Southern *rbcL* assay results with DNA extraction procedures performed under STANDARD laboratory conditions using bovine tissue

	Kidney	Liver	Spleen
Positive[1]	0	6*	10*
Inconclusive[2]	9	3	1
Negative[1]	2	2	0
Negative extraction control	-/- valid	+/- invalid	+/+ invalid
Negative PCR control	-/- valid	-/- valid	+/- invalid
Positive extraction Control	+/+ valid	+/+ valid	+/+ valid
Positive PCR control	+/+ valid	+/+ valid	+/+ valid

[1]Positive or negative = Double positive or negative PCR results

[2]Inconclusive = Single positive and negative PCR results

* Potentially false positive due to at least one invalid negative control

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this *rbcL* PCR assay, although nine of the 11 kidney samples yielded inconclusive (e.g. one positive and one negative) PCR results. However, many of the negative extraction buffer controls were also positive, an indication that contamination had occurred at sometime during DNA extraction and/or PCR, making it impossible to reach any valid conclusions from this experiment.
Several possibilities were identified as to the point at which contamination might have occurred:

1. One of the reagents used during DNA extraction was contaminated (unlikely, because the same reagents were used for all extractions and should have produced uniform bands across all samples)
2. The PCR itself was contaminated (also unlikely because only one of six negative controls was contaminated)
3. The contamination occurred during DNA extraction

In order to test the possibility that contamination might have occurred during DNA extraction, a second set of subsamples was homogenized and extracted in a laboratory where no plant DNA had ever been handled. Examination of the extraction controls prior to further analysis of the new DNA extracts showed that those were again contaminated, suggesting that aerosolized chloroplast DNA might be omnipresent in the facility and difficult to avoid.

A third DNA extraction was performed using all new reagents and all procedures were performed in another microbiology laboratory not previously exposed to plant DNA under a HEPA-filtered laminar flow biohood (Baker Co. Model VBM-400 and VBM-600) and using sterile gloves and a fresh lab coat to avoid accidental carryover of plant DNA into the lab. Using these precautions, the extraction controls were not contaminated. Table II shows the *rbcL* PCR assay results for the 33 beef organ tissue samples when working under these stringent controlled laboratory conditions that included performing all procedures under HEPA-filtered laminar airflow.

Unlike the previous set of results, only one out of the 11 liver samples and none of the 11 tested spleen samples were positive for the *rbcL* gene fragment by the conventional PCR assay. As before, none of the kidney samples were positive, although now only one of the 11 kidney samples yielded inconclusive results. The data in Table II shows that ten kidney, eight liver and ten spleen samples were truly negative for the *rbcL* gene fragment, although there were still four inconclusive results. Extractions for the inconclusive results were not repeated.

Determining the LOD of the TaqMan *rbcL* PCR assay

Genomic DNA and total RNA were isolated from beef and chicken spleen. Total RNA was converted into cDNA by reverse transcription and this cDNA, as

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Table II: Conventional PCR/Southern *rbcL* assay results with DNA extraction procedures performed in a laminar flow biohood under stringently CONTROLLED laboratory conditions using bovine tissue

	Kidney	Liver	Spleen
Positive[1]	0	1	0
Inconclusive[2]	1	2	1
Negative[1]	10	8	10
Extraction control	-/- valid	-/- valid	-/- valid
Negative PCR control	-/- valid	-/- valid	-/- valid
Spike	+/+ valid	+/+ valid	+/+ valid
Positive PCR control	+/+ valid	+/+ valid	+/+ valid

[1]Positive or negative = Double positive or negative PCR results

[2]Inconclusive = Single positive and negative PCR results

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well as isolated genomic DNA were assayed for the presence of nucleic acid encoding *rbcL* by TaqMan real time PCR, using two non-overlapping reagent sets for *rbcL*, (called RbcL_1 and RbcL_2) multiplexed with the internal animal house-keeping gene, 18S. Similar to the studies described for the conventional PCR, optimizations and subsequent limit of detection experiments were performed with the TaqMan PCR assay to assess the sensitivity of the assay before conducting analysis of the beef and chicken spleen samples. The two *rbcL* reagent sets were optimized using known amounts of *rbcL* amplicon. It was determined that both TaqMan PCR assays for *rbcL* reached a limit of detection of approximately two copies of this gene fragment per μl of reaction volume, comparable to the sensitivity of the conventional PCR combined with Southern blot analysis (data not shown).

Analysis of beef and chicken tissue samples with the TaqMan *rbcL* PCR assay

DNA encoding 18S was detected in genomic DNA from spleens of cattle or chickens fed a maize-rich diet. DNA encoding 18S was relatively abundant, exhibiting C_T values (the cycle number at which fluorescence generated by a reaction is significantly above the baseline signal, in this case it was set to the cycle number at which half maximal fluorescence was observed) ranging from 16.0-19.6 cycles (Figure 2).

In contrast, DNA fragments of *rbcL* were not detected in these samples, with either set of *rbcL* TaqMan reagents. C_T values for both the RbcL_1 and RbcL_2 primer sets ranged from 38 to 40, which is beyond the resolution limit of this

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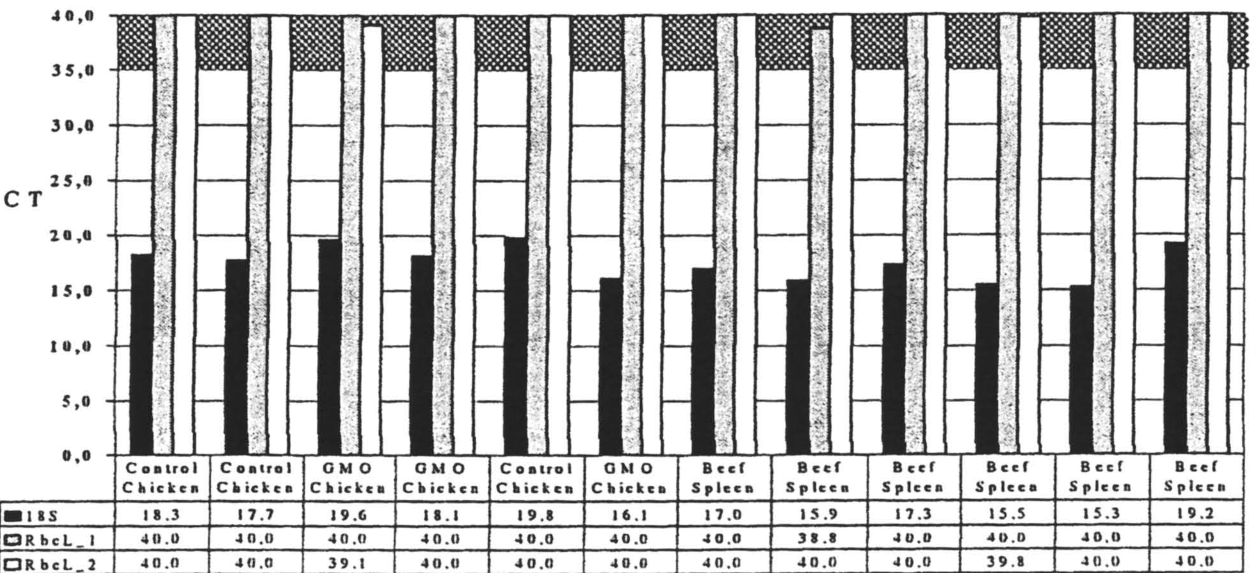

	Control Chicken	Control Chicken	GMO Chicken	GMO Chicken	Control Chicken	GMO Chicken	Beef Spleen	Beef Spleen	Beef Spleen	Beef Spleen	Beef Spleen	Beef Spleen
18S	18.3	17.7	19.6	18.1	19.8	16.1	17.0	15.9	17.3	15.5	15.3	19.2
RbcL_1	40.0	40.0	40.0	40.0	40.0	40.0	40.0	38.8	40.0	40.0	40.0	40.0
RbcL_2	40.0	40.0	39.1	40.0	40.0	40.0	40.0	40.0	40.0	39.8	40.0	40.0

Figure 2: Lack of detection of DNA fragments of the maize rbcL and animal 18S genes in beef and chicken spleen. Shown are the C_T values for 18S compared to RbcL_1 and RbcL_2. A C_T>35 is beyond the resolution limit and scored as a "negative" for detection. Data for each sample are the mean of six TaqMan reactions and two independent assays.

assay and more than 20 cycles higher than the C_T values for the 18S gene (Figure 2). This indicates that *rbcL* DNA was not present in these samples in amounts equal or greater than 1,048,576-fold (2^{20}-fold) less than the 18S gene fragment. In other words, maize *rbcL* was not detected in genomic DNA from these samples by two different, highly sensitive TaqMan PCR assays.

The potential expression of *rbcL* DNA fragments was also examined by RT-TaqMan PCR, more specifically, cellular RNA was isolated and converted into cDNA via reverse transcription (RT) and the presence of *rbcL* sequence was detected by TaqMan PCR. cDNA encoding 18S was detected in cellular RNA from spleens of cattle and chickens fed a maize-rich diet. cDNA encoding 18S was relatively abundant, exhibiting C_T values ranging from 15.3-19.8 cycles (Figure 3). In contrast, *rbcL* cDNA was not detected in these samples, with either set of *rbcL* reagents. C_T values for both RbcL-1 and RbcL_2 primer sets ranged between 38 to 40, beyond the resolution limit of this assay (Figure 3). The observed 18S signal was indeed the product of RNA since mock reactions lacking reverse transcriptase (RT- controls) yielded C_T values that were identical to background control reactions that did not receive template (NTCs produced C_Ts of 35-40). Therefore, fragments of transcripts of the plant *rbcL* gene were not detected in cellular RNA from these samples by two different, highly sensitive TaqMan assays.

Discussion

The present study describes two PCR methods for detection of fragments of the highly abundant plant chloroplast rubisco gene (*rbcL*). Conventional PCR/Southern blot and TaqMan real time PCR were both optimized for high sensitivity and specificity and used to attempt detection of this test plant gene in organ samples from cattle and chickens fed a maize-rich diet. Under stringent laboratory conditions that included conducting all DNA extraction and PCR procedures under HEPA-filtered airflow, it was not possible to detect significant traces of fragments of the plant-derived *rbcL* DNA in the tested animal tissue samples. However, if DNA extraction and PCR procedures were conducted under conventional laboratory conditions, the conventional PCR/Southern blot assay detected DNA fragments of the *rbcL* gene in almost all of the tested beef spleen samples and the majority of the beef liver samples. Clearly, the laboratory conditions under which these types of highly sensitive PCR assay methods are employed can have a dramatic effect on the potential outcome and interpretation of the results, especially if the proper positive and negative controls are not included throughout the experimental procedures to test for assay errors.

The results from the present conventional PCR studies conducted under stringent laboratory conditions match previously published data that showed lack of detection of fragments of highly abundant plant genes in bovine spleen and

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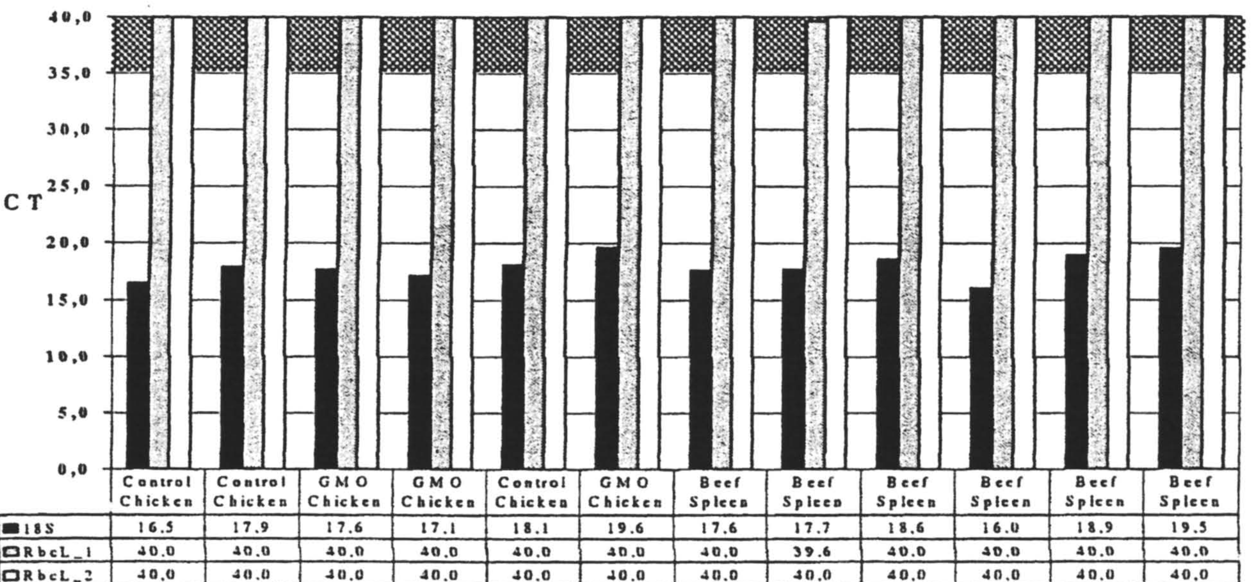

	Control Chicken	Control Chicken	GMO Chicken	GMO Chicken	Control Chicken	GMO Chicken	Beef Spleen	Beef Spleen	Beef Spleen	Beef Spleen	Beef Spleen	Beef Spleen
18S	16.5	17.9	17.6	17.1	18.1	19.6	17.6	17.7	18.6	16.0	18.9	19.5
RbcL_1	40.0	40.0	40.0	40.0	40.0	40.0	40.0	39.6	40.0	40.0	40.0	40.0
RbcL_2	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0

Figure 3: Lack of detection of rbcL and 18S cDNA (RNA) in beef and chicken spleen. Shown are the C_T values for 18S compared to RbcL_1 and RbcL_2. A C_T>35 is beyond the resolution limit and scored as a "negative" for detection. Data for each sample are the mean of six TaqMan reactions and two independent assays.

liver (12). The TaqMan PCR results showing no detection of fragments of the plant *rbcL* gene or the *rbcL* transcript in bovine spleen are also consistent with these previously published data. The lack of detection of this high copy number plant gene is remarkable for two reasons. First, it has been estimated that a 600 kg animal, such as a cow, ingests approximately 0.6 grams of DNA on a daily basis (13). This amount of consumed DNA would include approximately 10^{14}-10^{15} copies of chloroplast DNA, and yet in spite of this vast quantity of ingested DNA, no fragments were detected in bovine organ samples (kidney, liver, spleen) known to concentrate foreign materials. Second, previous studies with mice had reported detection of DNA fragments in circulating immune cells (6). Therefore, the present lack of detection of plant DNA fragments in bovine spleen, an organ rich in immune cells, shows that if immune cells in a ruminant animal take up DNA fragments, this must be a transient and/or a very rare event.

By comparison, the TaqMan PCR results with DNA extracted from chicken spleen samples are in contrast to formerly reported positive findings (12). One simple explanation for the different results could be the differences in analytical methodology. For instance, the DNA extraction methods varied (commercial column technique used in 11 versus EtOH precipitation and spooling used here). A second simple explanation is that the previous results showed detection of DNA fragments of the chloroplast $tRNA_{LEU}$ gene, while the present studies are with the chloroplast *rbcL* gene.

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Possible reasons that very sensitive PCR assays for high copy plant gene DNA fragments are susceptible to inadvertent contamination

Three factors may contribute to the high susceptibility of PCR assays to contamination artifacts that can occur during the DNA extraction or PCR procedures. One factor is that the present LOD experiments showed that it was possible to detect DNA fragments of the *rbcL* gene by the conventional PCR/Southern blot method down to 10 fg of input DNA. The TaqMan real time PCR had similar assay sensitivities. For both PCR methods, this level of sensitivity is approximately $1/100^{th}$ the level of a single nuclear haploid gene, or well below the sensitivity appropriate for single copy genes, requiring laboratory conditions during DNA extraction and PCR to be much more stringent than typically needed for studies of nuclear genes.

A second factor might be the fact that the chloroplast genome is significantly smaller than the nuclear genome. For instance, the size of the maize chloroplast genome is approximately 1.4×10^5 bp (GeneBank Accession No. X86563) which is more than four orders of magnitude smaller than the nuclear genome (~2.3-2.7×10^9 bp) (10), thus it might be more likely that chloroplast DNA is aerosolized than nuclear DNA such that under typical laboratory conditions the chloroplast DNA can contaminate DNA extraction or PCR reaction buffers.

A third factor might be that the chloroplast genome is more conserved throughout the plant kingdom than suspected, based on gene sequences currently found in public databases. The primers used in the present studies were designed to be maize specific when compared to *rbcL* gene sequences from other plant species found in public databases. However, only a small fraction of genes from the vast diversity of plant species found in nature have been sequenced to date. The maize *rbcL* gene may have unexpected homology to comparable chloroplast genes in other plant species found in the environment.

Considerations to avoid inadvertent sample contamination

The measures needed to avoid inadvertent environmental contamination of PCR assays screening for chloroplast DNA are very stringent, as shown by the present studies. To repeat the experiments described here, it should be taken into consideration that working in a laboratory where plant DNA is regularly analyzed carries a high potential for contamination. Even when working in a laboratory that does not routinely handle plant tissues, significant care is needed to avoid unintentional detection of environmentally derived chloroplast DNA fragments. Personal protective gear (e.g. sterile gloves, fresh lab coat) and performing the DNA extraction and PCR procedures within a HEPA-filtered laminar flow biohood were necessary in the present studies to obtain consistently negative buffer control results. Biological barriers such as using PCR primers purposely designed to be specific for the test chloroplast gene is also advisable, even though the present studies found widespread contamination under standard laboratory conditions even when using maize-specific PCR primer sequences.

Summary

The present study describes two PCR methods developed to detect fragments of the high copy number maize chloroplast rubisco *rbcL* gene. Conventional PCR/Southern blot and TaqMan real time PCR were both optimized for high sensitivity and specificity. The limits of detection for both *rbcL* PCR methods were shown to be in the low femtogram range of input DNA, which is actually below the theoretical limit of detection for single copy nuclear genes using these assays. Under stringently controlled laboratory conditions during DNA extraction and PCR for both methods, it was not possible to detect a fragment of the plant-derived DNA in beef kidney or spleen. One liver sample out of 11 tested positive for *rbcL*, but given the extreme caution needed to avoid accidental contamination by environmental factors, it is not possible to conclude that this single positive detection is biologically meaningful. The tested chicken spleen samples were all negative in the TaqMan assay, which is in contrast to formerly published results (12) with a similar focus but variations in the

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experimental approach. Working in standard laboratory conditions throughout DNA extraction and PCR, the conventional PCR/Southern blot results suggested substantial detection of the *rbcL* DNA fragment in almost all of the tested beef spleen samples and the majority of the beef liver samples. Detection of the *rbcL* DNA fragment in buffer-only controls showed these data were invalid. Therefore, it is clear that the laboratory conditions under which these types of highly sensitive PCR assay methods are employed can have a dramatic effect on the potential outcome and interpretation of the results. At a minimum, proper positive and negative controls are required throughout the experimental procedures to test for assay errors.

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Chapter 7

A Review of the Food/Feed Safety and Benefits of *Bacillus thuringiensis* Protein Containing Insect-Protected Crops

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Infestation of agricultural crops by insect pests has been traditionally managed through the use of chemical insecticides. An alternative method to control insect pests has been the introduction of insecticidal proteins from *Bacillus thuringiensis* into agricultural crops by genetic engineering. The introduced insect control proteins have an exemplary safety record having been safely used in agriculture for 40 years as the active ingredients of microbial pesticides. Insect-protected biotech crops control a variety of insect pests such as corn borers, cotton bollworms, and Colorado potato beetles. Season long protection of the crop improves yield and reduces reliance on traditional chemical insecticides. Protection of corn plants against insect damage reduces infection by certain fungal pathogens that produce fumonisin mycotoxins that are toxic to various species.

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Introduction

Biotechnology has made it possible to develop new plant varieties with improved agronomic properties such as *in planta* protection against insect pests. The gene(s) encoding the insect control proteins present in *Bacillus thuringiensis* (*Bt)*microbial pesticide products have been introduced into important food/feed crops such as corn, cotton and potatoes (*1,2,3*). *Bt* microbial pesticide products have been used commercially for almost 40 years by growers to control selected insect pests and have an exemplary safety record (*4*). *Bt* derived insect control proteins expressed *in planta* protect against insect pests that feed on plant tissue improving the health of the plant. Plants damaged by insects are more susceptible to infection by pathogens such as fungi. Certain pathogenic fungi elaborate toxic metabolites such as fumonisin mycotoxins in corn kernels that can make the grain less safe for consumption by humans and farm animals. Insect-protected plants such as *Bt* cotton also require less insecticide application to control lepidopteran pests reducing farm applicator exposure and insecticide release into the environment.

This chapter describes the safety assessment of insect-protected food/feed crops including information on the safety of the *Bt* insect control proteins. Results of animal feeding studies complement findings from extensive agronomic and nutrient compositional studies establishing the comparability of insect-protected plants to non - insect protected crop varieties. The benefits of insect protected crops will also be discussed.

Bt-Protected Plants

Bt-protected crops have been genetically modified to produce insect control proteins in plant tissues. *Bt* is a ubiquitous gram-positive soil bacterium; there are more than eighty subspecies of *Bt* that have been identified (*5,6*). *Bt* subspecies used as microbial pesticides were originally isolated from dead insect larvae (*7*). *Bt* subspecies form crystalline (Cry) protein inclusions (parasporal bodies) during sporulation (*8*). The Cry insect control proteins are principally active against lepidopteran, dipteran or coleopteran pests.

Bt microorganisms have been produced by fermentation and formulated as topically applied microbial pesticides that are sprayed on the surfaces of plants, soil, water environments and food storage facilities to control insect pests (7). The first *Bt* microbial pesticide formulation was approved for use in 1961. It was based on *Bt kurstaki* strain HD1, which produces four Cry proteins that are active against lepidopteran pests; Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa. The *Bt* microbial pesticide formulations have an exemplary safety record following 40 years of use in agriculture (9). There are currently at least 180 registered

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microbial *Bt* products (10) and over 120 microbial products in the European Union. Microbial *Bt* formulations are exempted from tolerances for application to human food crops to control insect pests and to drinking water to control mosquito larvae. *Bt* microbial pesticide formulations account for about 1 to 2 percent of the global insecticide market (*4*). It is estimated that 13,000 tons of *Bt* microbial formulations are produced by fermentation each year (*7*).

The efficacy of *Bt* microbial formulations topically applied to the surface of leaves is limited as the Cry proteins can be inactivated by sunlight or washed off by rain. Therefore, season long protection against insect pests cannot be practically achieved with surface applied *Bt* microbial pesticide formulations. However, if the Cry proteins could be produced *in planta,* then season long protection against susceptible insect pests would be possible. During the late 1980's, the first *cry* genes were cloned and expressed in tomato, tobacco and cotton plants (*1,2,3*). Today, *Bt*-protected potato, cotton and corn have been commercialized in the United States and one or more of these products are grown in Argentina, Canada, China, India, South Africa, and the Ukraine (*11,12*). There are more than 100 *cry* genes described (*13*) and there is significant potential for expanding the role of *Bt* mediated plant protection.
"The next generation of *Bt*-protected plants will contain multiple *cry* genes, thereby providing growers with a product that offers a broader spectrum of pest control and reduced susceptibility for insects to develop resistance."(*9*)

Safety Assessment

Mode of Action

Microbial *Bt* pesticide formulations contain full-length Cry proteins (130-140 kDa.) and smaller molecular weight forms of the full-length Cry protein generated by microbial proteases. Following ingestion by larval insects, the Cry protein is solubilzed in the alkaline (pH 8-10) juices of the insect midgut and proteolyticallyally processed to an insecticidally activate form (60-70 kDa). For many Cry proteins, activation occurs by cleavage of 26-28 amino acids from the N-terminus and about 600 amino acids from the C-terminus of the full length Cry protein molecule (*8*). The activated Cry insect control protein passes through the peritrophic membrane and binds to specific, high affinity, glycoprotein binding sites on the apical microvillar brush border membrane of midgut epithelial cells. After binding, the Cry protein is partitioned into the brush border membrane

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forming ion-selective channels in the cell membrane (*14*). The epithelial cells swell due to an influx of water resulting in cell lysis. Electrolyte imbalance occurs in the hemolymph, the insect stops feeding and dies (*15*). Binding to high affinity receptors is an essential step in the intoxication process.

In susceptible insects (e.g., lepidoptera), the toxicity of a particular *Bt* protein is correlated with the number of high affinity binding sites on the microvilli of gut epithelial cells (*14, 16, 17, 18*). The absence of toxicity of Cry proteins to non-target invertebrate species (*7*) may be due to the absence of high affinity Cry protein binding receptors on gut epithelial cells. Insects that are not susceptible to Cry proteins do not exhibit high affinity binding sites (*17*). Several studies with vertebrate species (mice, rats, monkeys, humans) have also failed to find high affinity Cry protein binding sites on gut epithelial cell membranes (*19,20*). This would explain why Cry insect control proteins are acutely toxic to susceptible insects with high affinity binding sites (ED50's of approximately μg/kg body weight dosages) but are non toxic to vertebrate species even when dosed at greater than 1 x 10^6 μg/kg body weight (*21,22*). Other considerations that support the dietary safety of Cry proteins for humans and animals are (1) the acidic environment of the mammalian stomach that does not favor solubilization and activation of the Cry proteins; (2) the rapid degradation of the Cry proteins by proteases in the mammalian gastrointestinal tract and (3) the lack of high affinity Cry-specific binding receptors on mammalian gut epithelial cells.

Insect protection traits

As stated earlier, the first commercial *Bt* microbial pesticide formulation was based on *Bt kurstaki* strain HD1 that produces four Cry proteins: Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa. These Cry proteins are active against lepidopteran pests. The *cry1Ab* and *cry1Ac* genes in the *Bt* HD1 strain are the prototypes for the genes currently expressed in corn and cotton to provide *in planta* protection against lepidopteran pests. *In planta* production of these insect control proteins confers insect protection throughout the growing season. Plant tissue levels of Cry proteins required to provide protection against pests are low, generally in the parts per million range. For example, in YieldGard ® corn, the levels of Cry1Ab protein range from 9-12 ppm in leaves to 0.3-0.5 ppm in the grain (*23*). In BollGard ® cotton, the levels of Cry1Ac protein in cottonseed range from approximately 5 to 20 ppm.

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Safety Assessment of the Cry Insect Control Proteins

In the United States, microbial *Bt* formulations containing Cry insect control proteins are regulated by the Environmental Protection Agency (EPA), which has regulatory jurisdiction over pesticides. The EPA also regulates genetically modified plants that produce Cry proteins *in planta* to provide insect protection. Before the mode of action of Cry proteins was well established, numerous toxicology studies were carried out to assess the safety of *Bt* microbial pesticide formulations for mammals and other non-target organisms.

These studies included acute toxicity and pathogenicity studies where large dosages of the *Bt* microbial pesticide formulations were administered by enteral and various parental routes of exposure (*9*). Subchronic and chronic feeding studies were also carried out with *Bt* microbial pesticide formulations (*9*). Findings from these studies are relevant to the safety assessment of *Bt*-protected plants because the microbial preparations contain the same classes of Cry proteins (Cry1, Cry2 and Cry3) introduced into plants.

Acute oral toxicity studies conducted in rats and rabbits found no mortalities at the highest doses tested, which ranged up to thousands of milligrams of *Bt* microbial formulation/kg bodyweight. No mortality, or dose related changes in body weight and food consumption and gross pathology at necropsy were observed in these studies (*22*). Subchronic and chronic feeding studies in rats demonstrated no findings of toxicological concern up to the highest dosage tested, 8,400 mg *Bt* microbial formulation/kg body weight/day (*22*). In two separate studies, human volunteers have been fed 1000 mg of *Bt* microbial preparations per day for up to five days and exhibited no symptoms of toxicity or other adverse effects (*22, 30*). The *Bt* microbial preparations used in the human feeding studies contained genes encoding the following Cry proteins, Cry1Aa, Cry1Ac, Cry1Ab, Cry1B and Cry2A.

Certain *Bt* subspecies also produce other non-Cry toxins that contribute to insecticidal activity. One of these is β-exotoxin which is not a protein, but a heat stable nucleotide, composed of adenine, glucose, and allaric acid. It inhibits RNA polymerase enzymes by competitive inhibition of ATP and is therefore non-selective in its toxicity (*7*).

The potential presence of this non-selective toxin in certain *Bt* subspecies prompted the EPA to require high dose acute toxicity testing of *Bt* microbial pesticide formulations in rodents to confirm its absence. The presence of β-exotoxin in commercial *Bt* microbial pesticide formulations is prohibited by EPA (*22*).

Other non-specific toxins are known as Cyt (for cytotoxic) protein toxins. There are four Cyt proteins known, Cyt1A, Cyt2A, Cyt1B, and Cyt2B (*13*). They are synthesized during sporulation, as are Cry proteins. Cyt proteins are much smaller than Cry proteins; the Cyt1A protein from *Bt* subspecies

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israeliensis is approximately 27 kDa. Cyt proteins are not structurally or functionally related to Cry proteins (*8,24,25*). They are proteolytically activated in the gut of insects and do not apparently require a high affinity receptor for binding to gut cells. They possess detergent-like activity disrupting the lipid and protein components in the membrane leading to cell lysis (*26,27,28,29*). This activity is non-specific as Cyt toxins can lyse both invertebrate and vertebrate cells *in vitro*. Others have also reported that certain *Bt* subspecies also produce hemolytic and non-hemolytic toxins and a diarrheal toxin (*30*). These toxins can be produced in a few *Bt* subspecies since *Bt* is closely related to *B cereus,* which can produce emetic and diarrheal toxins that cause food poisoning.

The presence of other, non-Cry toxins in certain *Bt* subspecies may be relevant to understand the results of a study in mice fed potatoes immersed in spores and crystals of *Bt* subspecies HD14 as well as transgenic potatoes containing a "Cry1" gene (Cry protein unidentified) (*31*). The authors reported microscopic changes in the luminal microvilli of mouse intestines, which were apparent in mice fed the potatoes dipped in *Bt* HD14 microbes. Mice fed the transgenic potatoes appeared similar to control mice fed non-transgenic potatoes. The authors did not correctly identify the subspecies that this *Bt* microbe belonged nor was any information provided to ascertain the dosages delivered to the mice (*30*). It was possible that the microbial preparations contained the β-exotoxin, which is known to damage intestinal cells. These findings are inconsistent with the "numerous studies that were used for the registration of *Bt* products that included microscopic pathologic observations of preserved tissues and no significant changes occurred" (*30*).

None of these aforementioned non-specific toxins found in certain *Bt* subspecies are produced in food and feed crops that have been genetically modified because only the gene for the specific Cry insect control protein is introduced into the plant.

Evolution in Safety Testing of *Bt* Microbial Pesticide Formulations and Insect Protected Food/Feed Crops Containing Cry Proteins

The safety testing requirements for registration of *Bt* microbial pesticide formulations has evolved over the years based on a comprehensive EPA review of completed toxicity/pathogenicity studies in 1982, in 1989 and again in 1998 (9,*10,21*). The elucidation of the highly selective mode of action of the Cry proteins has also contributed to this evolution. "While subchronic and chronic safety studies were conducted with the first *Bt* microbial products that were developed, the EPA has subsequently decided that acute hazard assessment is sufficient to assess the safety of new *Bt* microbial products. This decision is

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based on the fact that Cry proteins in *Bt* microbial products act through acute mechanisms to control insect pests, and these mechanisms are not functional in man" (*9*). "A battery of acute toxicity/pathogenicity studies is considered sufficient by the Agency to perform a risk assessment for microbial pesticides. Furthermore, the *Bacillus thuringiensis* delta-endotoxins affect insects via a well-known mechanism in which they bind to unique receptor sites on the cell membrane of the insect gut, thereby forming pores and disrupting the osmotic balance. There are no known equivalent receptor sites in mammalian species that could be affected, regardless of the age of the individual. Thus, there is a reasonable certainty that no harm will result to infants and children from dietary exposures to residues of *Bacillus thuringiensis*" (*21*).

Following their review of all the submitted toxicology studies on *Bt* microbial pesticide formulations, EPA scientists concluded "Toxicology studies submitted to the U.S. Environmental Protection Agency to support the registration of *B. thuringiensis* subspecies have failed to show any significant adverse effects in body weight gain, clinical observations or upon necropsy." (*22*).

In a WHO IPCS Environmental Health Criteria document on *Bacillus thuringiensis*, similar conclusions regarding the safety of *Bt* microbial pesticides were made. A panel of internationally recognized experts reviewed extensive literature regarding safety assessments of microbial *Bt* pesticide formulations and concluded "Owing to their specific mode of action, *Bt* products are unlikely to pose any hazard to humans or other vertebrates ..." and " *Bt* has not been documented to cause any adverse effects on human health when present in drinking water or food" (*7*).

For safety assessment of Cry proteins expressed *in planta*, acute toxicity testing along with digestibility testing *in vitro* is considered appropriate and sufficient to assess health risks from dietary exposure to Cry proteins (*32*). Pathogenicity and infectivity testing, which has been conducted with viable *Bt* microbes in pesticide formulations would be inappropriate for Cry proteins. Dermal, ocular and inhalation exposure testing is not normally required because farm worker exposure to Cry proteins expressed in plants is believed to be negligible. In plants, Cry proteins are expressed at low levels (ppm) and largely contained within the cells of the plants.

As a condition for EPA registration, Cry insect control proteins introduced into plants have been administered acutely at very high dosages to laboratory rodents as part of an overall hazard assessment. The dosage levels administered to rodents are generally in the 1000's of mg/kg range. To date, no adverse effects have been observed in rodents dosed acutely with Cry insect control proteins introduced into plants. This provides extremely large margins of safety for humans or farm animals that might consume corn grain containing these Cry proteins. For example, a 600 kg dairy cow would have to eat 4,800,000 kg of

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YieldGard corn grain containing 0.5 ppm Cry 1Ab protein to equal the 4000 mg/kg Cry 1Ab protein dose given acutely to mice. Similarly, a 60 kg human would have to eat 480,000 kg of YieldGard corn grain to achieve the same dose of 4000 mg/kg Cry1Ab protein given to mice. The 4000 mg/kg Cry1Ab dose produced no toxic effects in mice (23). Based on the absence of mammalian toxicity for Cry1Ab protein and the large margins of safety for dietary exposure, it is concluded that Cry1Ab protein poses no meaningful risk to human or animal health.

Digestibility of Cry proteins

Conventional chemical insecticides are not normally degraded in the gastrointestinal (GI) tract and can be absorbed systemically, although the potential for systemic exposure is generally quite low since the residual levels found on food crops are well below tolerance limits. However, the potential for systemic exposure to a Cry protein in a food crop would be even lower since it would be degraded in the GI tract like other dietary proteins. The digestive systems of humans and animals are designed to effectively degrade intact dietary proteins into their constituent amino acids and small peptides.

The digestibility of Cry1, Cry2 and Cry3 proteins has been tested *in vitro* using simulated mammalian gastric fluids (*33,34,35,36,37,38*). These proteins, which are typically 60 to 130 kDa in size, are readily degraded (often in less than 30 seconds) in simulated digestion fluids to polypeptides of less than 2 kDa that correspond to peptides less than 10 amino acids in length. These *in vitro* models are significantly less robust than the intact gastrointestinal systems of humans and animals, which suggests that the Cry proteins will be rapidly and extensively degraded following consumption.

No residues of Cry proteins have been detected in tissues of animals fed grain containing these proteins. For example, no intact or immunologically reactive fragments of the Cry1Ab protein were detected in pork loin muscle tissue from grower-finisher pigs fed *Bt* corn (YieldGard®) (*39*). In another study, no Cry protein was detected in the milk of lactating dairy cows fed green chop from insect-protected *Bt* corn (*40*).

Allergenicity assessment

A unique hazard posed by a small number of proteins naturally found in foods is the potential to sensitize genetically susceptible individuals causing

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allergic reactions when the offending proteins are consumed. The reactions may range in severity from mild (skin rash, GI upset) to life threatening anaphylaxis, which fortunately occurs infrequently. The process of assessing the potential allergenic risks of proteins introduced into food crops has been developed by the U.S. Food and Drug Administration and further modified by allergy experts at FAO/WHO scientific workshops on allergy assessment (*41,42,43*). The assessment involves comparing the structural and biochemical properties of the introduced protein to known protein allergens. This can be done, in part, by comparing the amino acid sequence similarity of the introduced Cry protein against known protein allergens in publicly available sequence databases using the FASTA sequence alignment tool (*44*). For more details on the sequence alignment comparisons, see Chapter 10 in this book. To date, none of the Cry proteins introduced in food/feed crops has shown any significant amino acid sequence similarity to any known protein allergens (*34-36,38 45-47*).

Comparison of the biochemical properties of Cry proteins to known protein allergens includes their stability to the low pH environment found in the stomach and to pepsin digestion. Proteins that are consumed first enter the stomach where they are degraded by the combined action of low pH and pepsin proteolysis. Known protein allergens tend to be stable to low pH and pepsin, while non-allergenic proteins tend to be unstable and readily degraded. This has been confirmed using simulated gastric fluid *in vitro* comparing the digestibility of non-allergenic proteins to known protein allergens (*38,45*). While not all stable proteins are allergens, the protein must remain stable long enough in the GI tract to elicit sensitization and subsequently, allergic reactions. In addition, proteins that are food allergens tend to be present in the food in abundant quantities, increasing the potential to cause sensitization and subsequent allergic reactions; in contrast, Cry proteins are present at low (ppm) levels in food.

Cry1, Cry2 and Cry3 classes of proteins that have been introduced into food and feed crops have been shown to be readily digestible (generally 30 seconds or less) when incubated *in vitro* using simulated gastric fluid (*34-36,38,46-49*).

The importance of digestibility assessments was recently illustrated by the failure of Aventis Crop Science Company to gain registration approval for the use of Cry9C in corn intended for use in human food. When tested in simulated digestive fluids, the Cry 9C protein was less digestible than the aforementioned class Cry1, Cry2, and Cry3 proteins. This raised concerns that it might be potentially allergenic, although, the risk seemed low, given the low levels of Cry9C protein that would have been present in corn grain. Nevertheless, this episode illustrates the conservative approach that is being used to assess the potential allergenicity of proteins considered for introduction into food plants.

Despite the evidence that Cry proteins do not fit the profile of known protein food allergens, there are reports in the literature that the Cry1Ac protein (used to protect cotton plants) is immunogenic. In a series of studies,

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investigators were examining the potential of Cry1Ac protein to induce systemic and mucosal immunity for the purpose of developing better oral vaccines (*50-52*). Cry1Ac protein was administered to mice by both oral and intraperitoneal (IP) routes of exposure and increased levels of IgG, IgM and IgA antibodies were found. This phenomenon, however, is not unique to the Cry1Ac protein, but is an antibody response that can be elicited following exposure to other non allergenic dietary proteins (*53-55*). All proteins have the ability to be immunogenic, but very few immunogenic proteins are allergens. These antibody responses are not associated with hypersensitivity since IgE, the major antibody responsible for allergic reactions, was not measured in this study. The study reported that Cry1Ac protein could increase intestinal IgG levels when administered as an adjuvant by IP injection, but not by the intragastric route. The IP route is very effective in inducing both systemic and mucosal immunity for most proteins because the peritoneal cavity is known to contain plasma cells destined for the mucosal tissues (*56*). Thus, the findings of increased intestinal IgG following IP administration have no particular relevance to dietary exposure. The doses of Cry1Ac protein administered by investigators to mice by enteral or parental routes were very high compared to potential human exposures to Cry proteins that might be consumed in a crop such as corn. For enteral exposure, the investigators used large amounts of a buffering agent to neutralize stomach acids and lessen proteolytic activity against the Cry1Ac protein. The combination of very high doses and interference with normal digestive processes increased the likelihood that some Cry1Ac protein would survive intact and be taken up by gut associated lymphoid tissue (GALT). The sampling and immune response to Cry1Ac protein is a normal response to any dietary protein, and is part of the process of development of oral tolerance whereby the GALT reduces the immune response to dietary proteins. In food allergy, normal tolerance for some reason fails to develop, and the GALT stimulates an allergic IgE response to the dietary protein which is normally reserved as a host defence against proteins produced by harmful pathogens or parasites (*57*).

Following review of the data in these studies, EPA scientists concluded that these series of studies "cannot be extrapolated to address the potential food allergenicity of Cry1Ac" (*57*).

Another report found increased circulating IgG and IgE antibodies to components of *Bt* microbial sprays in a subset of workers who applied *Bt* microbial formulations to agricultural crops (*58*). However, there were no reports of clinical allergic disease in the workers or of serum antibodies to the Cry protein components of the microbial formulation (*57*). EPA scientsist concluded after reviewing the results of this study that "The fact that this subset of workers showed signs of exposure to the *Bt* sprays without developing an allergic response is significant and adds weight to the finding that *Bt* delta-endoxin (Cry) proteins considered here are not expected to induce an allergic

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reaction" (*57*). The results of this study are consistent with the absence of allergic reactions in workers who have either produced or used *Bt* microbial sprays which contain various Cry proteins "After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports that of immediate or delayed allergic reactions to the delta-endoxin itself, despite significant oral, dermal, and inhalation exposure to the microbial product" (*57*).

Animal feeding studies

There is a comprehensive body of data to establish the safety of Cry proteins introduced into food/feed crops. Concerns have been raised about the potential for "pleiotrophic" or unintended changes in the genome resulting from insertion of the introduced gene(s) into the plant genome.

In conventional breeding of plants, many genes are introduced into the plant genome during breeding. Sometimes the progeny do not develop normally. These "off-types" are generally detected during evaluation of the agronomic properties of the progeny in the greenhouse and/or the field. "Off-type" varieties are generally discarded, as they do not possess the agronomic properties needed for commercialization. The same evaluation procedures are followed when new crop varieties are developed through biotechnology. Only those progeny with the desired agronomic properties are advanced to commercialization. Biotech crops are also subjected to more evaluation than conventional bred crops in that a comprehensive compositional analysis is performed looking for unintended changes in important nutrients and antinutrients that may be naturally present in the food crop. The results of comprehensive analysis of agronomic properties and composition of biotech crops containing Cry proteins have been published (*23,59*). There have been no unintended changes detected in the agronomic properties or composition of crops with introduced Cry insect control proteins. Despite these data, concerns about the safety of these crops continued to be raised. Animal feeding studies were subsequently undertaken to provide further assurance of the safety of the biotech crops for human and farm animal consumption.

As the adoption of biotech crops grew between 1996 to 2000, the animal production industry requested that performance studies be carried out with farm animals fed biotech crops. The primary issue was to affirm that palatability was unchanged and that farm animals fed insect-protected *Bt* crops (including event MON 810 producing the Cry1Ab protein) performed as well as animals fed conventional varieties. Numerous studies with beef and dairy cattle, broiler and layer chickens, swine and sheep have shown there is no meaningful difference in

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performance when animals are fed biotech crops compared with animals fed conventional crops *(60-66)*.

Safety assurance studies for human consumption of biotech crops have also been carried out in laboratory rats, a species that have been used historically to assess the safety of substances added to human food. Male and female rats (20 rats/sex/group) have been fed grain from *Bt* corn and control (non transgenic corn) in the diet at levels up to 33 wt%, representing the concentration of corn normally added to commercial rodent diets (*67*). Rat diets were formulated to contain test (biotech corn) or control corn (control hybrid with background genetics similar to the test hybrid but lacking the *cry1Ab* gene) that were grown at the same time and in the same fields. Additional groups of rats (reference controls) were fed corn grain from 6 different non-transgenic commercial corn hybrids in an attempt to define the normal range of responses of rats fed different corn hybrids. No one has established this normal range of response since corn hybrids developed from conventional breeding techniques are not normally fed to rats to confirm their safety. The 10 groups of rats (a total of 400) were fed diets for 90 days and general health and appearance, weekly body weights and food consumption, clinical parameters (hematology, blood chemistry, urinalysis), organ weights, gross and microscopic appearance of tissues were evaluated.

Results of the rat feeding study found that the aforementioned parameters were comparable for rats fed the test and control corn grain (*67*). These results are consistent with those of previous farm animal feeding studies (*68*). Some of the farm animal feeding studies with *Bt* corn also have relevance to human food safety. Swine are good models for humans given the similarity of their digestive and cardiovascular system to that of humans. The poultry feeding study confirmed the nutritional comparability of *Bt* corn to conventional corn varieties. Day 1 chicks increase their body weight 50 to 65 fold over the 6 week study period and are sensitive to nutritional deficiencies in their food given their rapid rate of growth.

Benefits

Growers sustain billions of dollars in crop loss or reduced yield due to pests that have the potential to be controlled by Cry proteins (*69*). The European corn borer causes stalk damage when second generation borers enter the corn stalks. Once in the stalk, corn borers are difficult to control with externally applied insecticides. The situation with cotton and bollworms is similar. Once the worms enter the cotton boll, they are very difficult to control.

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Corn plants that are stressed by insect damage are more susceptible to fungal infection. Certain fungal species such as *Fusarium verticillioides* and *Fusarium proliferatum* can be found in corn wherever it is grown around the world. These fungi produce secondary metabolites or mycotoxins known as fumonisins. Fungi can be introduced into the corn plant by corn borers feeding on stalk and ear tissue. The resulting damage to developing grain enables spores of the toxin-producing fungi to enter the kernel and proliferate producing corn ear rot. The fungi produce fumonisins, which can make the grain unhealthy for human or animal consumption. At levels greater than 10 to 20 ppm in grain, fumonisin contamination can cause death or morbidity in horses and swine respectively (*70*). Epidemiological studies have linked consumption of corn containing high levels of fumonisins with an elevated incidence of esophageal and liver cancer in African farmers (*71*). Fumonisins have been shown to cause liver and kidney cancer in rodents. As a consequence of growing concerns about the potential health implications of fumonisins, and their ubiquitous presence in corn grown around the world, international health agencies (FAO/WHO JECFA etc.) have established a provisional maximum tolerable daily intake (PMTDI) of 2 ug fumonisin per kg body weight dietary exposure for humans (*72*). Guidance levels for fumonisin contamination in grain have been set by two countries, Switzerland (1 ppm) and the United States (3-4 ppm for human food, 5 ppm for horses, 20 ppm for swine, and higher levels for poultry and ruminants) (*73*). For horses and swine, the contaminated corn cannot exceed 20% of the diet mix so fumonisin exposure to animals is further diluted.

Since insect damage caused by corn borers can predispose plants to fungal growth and fumonisin contamination, protection against corn borers can, in some environments, significantly reduce fungal and mycotoxin contamination. Munkvold et al. (*74,75*) were the first to show that *Fusarium* ear rot and fumonisin contamination were significantly reduced in insect-protected *Bt* corn compared with non-*Bt* corn carried out in field trials over several seasons. This was confirmed in additional field trials carried out with insect-protected *Bt* corn grown over 3 years in Illinois (*76*) as well as field trials carried out in the United States, France, Spain and Italy (*77-79*). All of these studies showed a reduction in fumonisin levels in the grain of *Bt* corn hybrids when compared with non-*Bt* corn hybrids (Table 1). Protection of corn plants against corn borer damage can prevent plant injury, reducing the potential for fungal and fumonisin contamination, which may improve the safety of corn grain for human and animal consumption.

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Table 1. Fumonisin Reduction in the Grain of *Bt* Corn Hybrids*

Country	Year	Field Sites	Mean Fumonisin Levels** Non *Bt*	*Bt*	Reference
Italy	1997	3	19.8	2.0	*77*
Italy	1998	4	31.6	5.4	*77*
Italy	1999	30	3.9	1.4	*77*
France/ Spain	1999	5	0.4 - 9.0	0.05-0.3	*78*
U.S	2000	49	3.07	1.36	*79*

* Monsanto corn hybrids containing Cry1Ab protein
** ppm total fumonisins averaged across field sites

In planta protection against insect pests can reduce the use of insecticides on the plant. Cotton plants used to be heavily sprayed with insecticides in the south and southwestern United States to control tobacco budworm, cotton and pink bollworm. Following the commercial introduction of insect-protected *Bt* cotton, there has been a significant reduction (millions of pounds annually) in the use of insecticides to control these pests (*69*). In China, where there has been widespread adoption of *Bt* cotton in the last few years, they have reduced the number of insecticide applications from 20 to 7 per hectare/season (*80*). This has reduced the insecticide cost by $762/hectare/season and has improved farmer health significantly since application of organophosphate and organochlorine insecticides has been decreased by up to 80%. The authors did not address the environmental benefits form the significant reduction of insecticide applications.

A new insect-protected *Bt* corn variety has been developed that provides protection against corn rootworm, a pest that causes significant damage to the roots of corn grown in the Midwest and Eastern United States (*81*). Since most of the insecticide applied to corn is intended to control the corn rootworm pest, the introduction of this new variety will result in a major reduction in the amount of insecticide applied to corn plants.

Conclusions

The advent of biotechnology has provided farmers with new tools to improve the production of healthy and wholesome food. *In planta* production of Cry insect control proteins that have an unblemished history of safe use in agriculture provide alternatives to the use of conventional chemical insecticides. The safety of Cry insect control proteins has been well established over the years based on extensive safety studies and widespread experience following its use in many countries. Season long protection of corn plants against corn borer pests

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can make the corn safer for human and animal consumption by reducing contamination with fungal pathogens that produce fumonisin mycotoxins in the grain. Comprehensive agronomic, compositional and animal feeding studies provide assurance of absence of significant unintended effects and confirm the safety of the grain for human and animal consumption.

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Chapter 8

Impact of *Bacillus thuringiensis* Corn Pollen on Monarch Butterfly Populations: A Risk Assessment

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A formal risk assessment of the impact of *Bt* corn pollen on monarch butterfly populations indicated that the impact was negligible. A collaborative research effort was undertaken by scientists in several States and in Canada. Information was sought on the toxic effects of *Bt* corn pollen and the degree to which monarch larvae would be exposed to active amounts of *Bt* pollen on its host plant, the common milkweed found in and around cornfields. Expression of Cry proteins, the active toxicant found in *Bt* corn tissues, differed among hybrids, and especially so in the concentrations found in pollen of different events. In most commercial hybrids *Bt* expression in pollen is low and laboratory and field studies show no toxic effects at any pollen density that would be encountered in the field. Other factors mitigating exposure of larvae include the variable and limited overlap between pollen shed and larval activity periods, the fact that only a portion of the monarch population utilizes milkweed stands in and near cornfields, and the relatively low adoption rate of *Bt* corn (approx. 35%) of North American corn-growing areas. This study identifies methodologies that could serve as a model approach to assessing the environmental impact of transgenic crops and illustrates the power of collaborative approaches to broader ecological questions.

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Introduction

Transgenic crops expressing Bt proteins have been grown commercially in North America for the past six years. Questions have been raised about their impact on non-target organisms and other possible effects on the environment such as genetic contamination and increased selection pressure on the target organisms. A particularly alarming report on the impact of Bt corn pollen on monarch butterfly larvae, ***Danaus plexippus*** L. (Lepidoptera: Danaidae) feeding on milkweeds in and near cornfields caused an uproar of controversy in Europe and subsequently in North America (*1*). In following months, a group of researchers with USDA and several Universities across the Corn Belt collaborated on research agendas and procedures to resolve the scientific concerns resulting from the publication of the short note cited previously. The research contributions reported here represent a collaborative effort established to specifically address the question of risk associated with *Bt* corn pollen to the monarch butterfly. In December 1999, the EPA issued a data-call-in requesting industry, researchers and all interested parties to submit information and comments by March 2001 for use in evaluation and potential re-registration of corn hybrids containing Cry proteins to which the effort reported here was a contribution.

In this paper, a weight-of-evidence approach is described for the risk of exposure of monarch larvae to *Bt* corn pollen and the impact of such exposure on populations of the monarch butterfly in eastern North America (2). Our conclusions are based on collaborative research by scientists in the United States and Canada (*3*-6). This approach to risk assessment has been performed for many non-target species in relation to pesticides (*7-10*), industrial by-products (*11-13*) and other potential toxicants found in the environment (*14*). The approach to this process is consistent, well documented and standardized. It requires consideration of both the expression of a toxicant and the likelihood of exposure to the toxicant as the basic components for a risk assessment procedure (*15*).

Materials and Methods

Toxicity of purified *Bt* proteins to larval stages of butterflies and moths is well known (*16,17*). Studies conducted on the use of *Bt* sprays in forests for gypsy moth control have shown that Cry proteins can adversely affect non-target Lepidoptera (*18,19*). Field data from these studies indicated a temporary reduction in lepidopteran populations during prolonged *Bt* use, although widespread irreversible harm was not apparent (*20*). Lepidopteran-active *Bt* protein expressed in pollen of *Bt* corn hybrids may pose a risk to sensitive species, such as monarch butterflies, in or near cornfields during anthesis

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(*1,21,22*). Milkweeds, *Asclepias* spp., and especially common milkweed, *A. syriaca* (L.), are the sole larval food source for monarch butterfly larvae and are abundant throughout the corn growing regions of North America (*23*). As such, hazard from *Bt* corn pollen deposited on milkweed leaves warrants consideration of its ecological risk to monarch populations.

Risk assessment requires knowledge of four essential components: 1) hazard identification, 2) nature of dose-response to a toxin, 3) probability of exposure to an effective dose, and 4) characterization of risk (*24*). Components of a risk assessment approach as applied to the case of *Bt* corn and monarch butterfly are depicted in Figure 1. *Bt* proteins expressed in corn plant tissues can bring about specific reactions in the gut of lepidopteran larvae (*25*) including non-target caterpillars that consume *Bt* corn pollen. The magnitude of the reaction will depend on the type of protein produced by various events of hybrid *Bt* corn, the amount of protein expressed in pollen grains from different events, the amount of pollen consumed by larvae of different developmental stages and the susceptibility of larvae to the *Bt* protein. That a hazard may exist was suggested by Losey *et al.* (*1*). Characterization of toxic effects is necessary to establish the first component of risk. Laboratory and field assays of lethal and sublethal toxicity resulting from exposure to doses of *Bt* pollen likely to be encountered are required to establish an effective environmental concentration (EEC), the toxicity threshold of *Bt* pollen in the environment. The EEC will vary based on expression levels for individual *Bt* corn events in conjunction with environmental factors determining ecological exposure.

Consideration of risk as a function of exposure and effect requires that lines of evidence be established in four areas of inquiry: 1) Is there some density of *Bt* pollen on milkweed leaves that represents a lethal or sublethal threat to monarch larvae or later stages of development? 2) What proportion of *Bt* pollen deposited on milkweed leaves in and around cornfields exceeds the EEC for larvae of monarchs? 3) What proportion of monarch populations utilize milkweed in and near cornfields? 4) What is the degree of overlap between the phenological stages of monarch larvae and corn anthesis over the shared range of these species?

Results

Cry1A proteins expressed in most commercial *Bt* corn hybrids are toxic to the monarch butterfly (Hellmich & Siegfried, 2001). Mortality, expressed as LD_{50}, was estimated at 3.3 ng protein/ml diet, while growth inhibition (EC_{50}) was estimated to be 0.76 ng/ml[2]. However, the expression of Cry1Ab endotoxin within pollen of various events varies considerably depending on the promoter gene involved (Christensen *et al.*, 1992). Expression is greatest in event 176 Bt corn (1.1 to 7.1 μg/gm pollen), a line that is being phased out. This event

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exceeds, by nearly two orders of magnitude, protein expression in events Bt11 and Mon810 (0.09 μg/gm pollen)), which is near the current level of detection by immunoassay.

Laboratory bioassays of pollen fed to neonate monarchs on leaf disks or whole, detached leaves of common milkweed, *A. syriaca*, indicate that pollen from event 176 *Bt* corn causes mortality and sublethal effects, such as growth inhibition, at very low concentrations (*3*). Growth inhibition, a more sensitive measure of protein intoxication, could be detected at <10 grains/cm^2. Pollen from all other events, including Mon810 and Bt11 corn hybrids as well as events not presently grown, such as Dbt418, Cbh351 and Tc1507 (expressing Cry1Ac, Cry9C, and Cry1F proteins, respectively), did not demonstrate any lethal or sublethal effects, even at densities above 1000 pollen grains/cm^2 (*3*). These data were used to establish a no-observable-effect-level (NOEL) for growth inhibition of larvae for event 176 pollen and for Bt11 and Mon810 pollen.

Five field bioassays were undertaken to determine the outcome of exposure of larvae under field conditions on milkweed plants growing or placed in the field. In Iowa, reduced weight gain was noted for larvae exposed to event 176 pollen on milkweeds within cornfields at densities of 20-25 pollen grains/cm^2. Both survival and weight gain were affected in Maryland, where a series of assays using milkweed leaves collected from plants in an event 176 cornfield were carried out over the pollen-shed period (*6*).

In a separate field trial in Maryland, the effects were evaluated on survival and growth of monarch neonates on leaves of milkweed within a field of a sweet corn hybrid expressing Bt11 endotoxin and compared with the effects of residues following applications of a pyrethroid insecticide. Survival of larvae that fed on insecticide-treated milkweed leaves from within the cornfield was low (0-10%). Survival also was influenced significantly (65-79%) by insecticide that drifted onto milkweeds leaves 3 m outside the field. In contrast, survival of larvae exposed to leaves taken from within both *Bt* and non-*Bt* corn plots ranged from 80-93%, and there were no significant differences in larval survival between these two plots (*6*).

Exposure to Bt corn pollen depends on 1) the phenological overlap between monarch populations and corn anthesis, 2) the spatial overlap between milkweeds used by monarchs and cornfields, and 3) the pollen densities encountered on leaves of milkweed plants in and near cornfields. Pollen from corn plants within a particular field is shed over a period of 7-15 days during the season, while larvae develop over a more prolonged period. Potential for exposure of susceptible stages of monarch larvae to corn pollen depends on synchrony of their development with pollen shed of corn plants. Locations in Iowa, Maryland, Minnesota/Wisconsin and Ontario were monitored for phenological development of monarch populations and anthesis (*4*). Overlap of the more susceptible stages of monarchs, primarily 1st and 2nd instars, with pollen shed was considered for purposes of risk assessment.

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Presence of susceptible larvae at the time of corn anthesis varied considerably across the regions studied (*4*). In the more northern locations (MN/WI and ON), about 40 and 62% of the larvae overlapped with pollen shed, respectively, while in areas further south (IA and MD), about 15 and 20% of the larval stages overlapped, respectively. Data from a computer simulation of monarch phenology and corn development support the general observation that overlap increases at higher latitudes across the Corn Belt[1].

Density of milkweed stands in cornfields compared with non-agricultural lands and data on the proportion of the landscape in corn and non-agricultural lands provided a basis on which to determine the proportion of the milkweed population that was in cornfields (*4*). In all locations, densities were higher in non-agricultural lands than in cornfields, but the range of difference was considerable. In Minnesota/Wisconsin and in Iowa, the density of milkweed was approximately 4-7 times greater in non-agricultural fields than in cornfields, while in Ontario the density was up to 115 times greater. In areas where corn is more intensively cultivated, as in Iowa and southern Minnesota/Wisconsin, less non-agricultural land exists and the overall proportion of milkweed on a landscape basis is higher in cornfields and other crop lands than in non-agricultural land. In regions of the corn growing area where mixed habitats are more common, such as in Maryland and Ontario, milkweeds are more abundant in the non-agricultural landscape and provide proportionately greater habitat than corn (*4*).

Dispersal of corn pollen was described by Raynor *et al.* (*26*), who demonstrated deposition of pollen as much as 60 m from field edges. During periods of pollen shed, samples of pollen were collected on sticky trap surfaces and on milkweed leaves at various distances within and beyond the margins of cornfields to estimate the concentration of pollen that could be encountered by monarch larvae (*5*). Data from three locations, Iowa, Maryland and Ontario demonstrated a 5-fold reduction in concentration of pollen from just within the edge of the cornfield to about 2-3m distant. Within-field densities across the different studies averaged between 65-425 pollen grains/cm^2 on milkweed leaves at the peak of corn anthesis with an average of 171 grains/cm^2.

To determine risks to monarch larvae associated with *Bt* corn pollen, two components of greatest significance are: 1) the frequency with which effective environmental concentrations (EEC) exceed the thresholds for mortality or sublethal effects, such as growth inhibition, of each *Bt* pollen type, and 2) the proportion of monarch larval populations in eastern North America that are exposed to toxic levels of *Bt* pollen.

It is clear from both laboratory and field-based studies (*3,6*) that pollen from the dominant commercial *Bt* corn hybrids (Mon810 and Bt11) does not express

[1] Pers. comm., D. Calvin, Department of Entomology, Pennsylvania State University, University Park, PA 16802.

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Cry1Ab protein to a level that will impact monarch populations to any significant degree. Hellmich *et al.* (*3*) suggested a conservative LOEC be established for these hybrids at 1000 pollen grains/cm^2 of milkweed leaf surface based on a combined analysis of laboratory bioassays exposing larvae to 1000-1600 of pollen grains/cm^2. Growth inhibition was evident for larvae exposed to event 176 pollen at 5-10 grains/cm^2, the lowest dose where activity was noted by Hellmich *et al.* (*3*), therefore the EEC for event 176 corn pollen will frequently exceed this threshold in fields where it is planted.

Probabilities of toxicity for events 176, Bt11 and Mon810 pollen are depicted in Figure 1 as a dose-effect relationship for exposure of larvae to pollen plotted on log-probability scales following methods accepted by EPA. Growth inhibition of monarch neonates in response to increasing concentrations of event 176 pollen, as reported by Hellmich *et al.* (*3*), is illustrated, with a no-observable-effect-level (NOEL) at 5-10 pollen grains/cm^2. In comparison, a hypothetical response curve for Bt11 and MON810 pollen is depicted using the same slope parameter for the event 176 response (the Cry1Ab protein is identical in each event), and with a lowest-observable-effect-concentration (LOEC) established, for sake of argument, as a range between 1000-4000 grains/cm^2. Pollen deposition on milkweed leaves during 1999-2000 (*5*) is represented on a separate scale in a cumulative frequency occurrence curve.

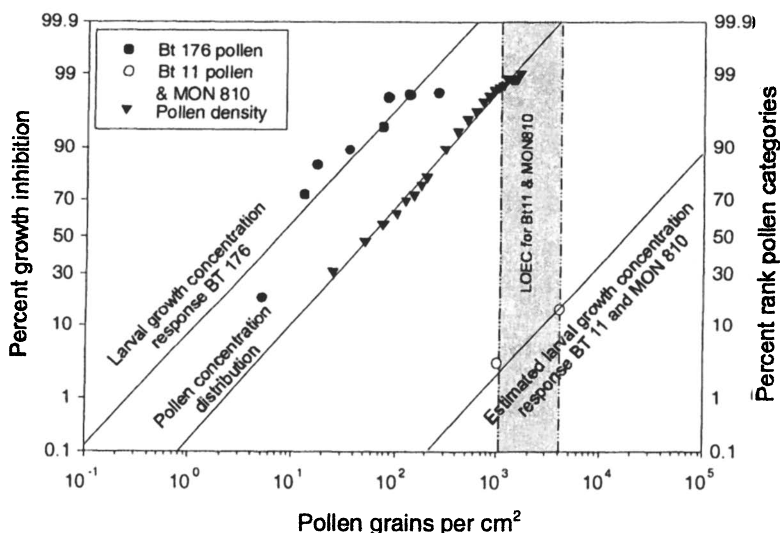

Figure 1. Percent growth inhibition of monarch larvae

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It is apparent from Figure 1 that significant overlap of commonly encountered concentrations of pollen from event 176 hybrids with doses necessary for growth inhibition (and mortality) would likely occur within or near cornfields. Ninety percent of the samples of milkweed leaves examined in our study of pollen deposition had a density at or above this level. By contrast, the LOEC range for Bt11 and Mon810 of 1000-4000 pollen grains/cm^2 would be encountered by larvae in only 0.7-0.1% of natural in-field situations. Pollen deposition estimated from any single sample over two seasons never exceeded 1600 grains/cm (*3,5*). Even if the LOEC for Bt11 and Mon810 were established at the conservative concentration of 1000 grains/cm^2, 99.3% of encounters by monarch larvae of pollen would be below this concentration (*5*).

Milkweeds exist in cornfields across most of the North American corn-growing regions and monarch butterflies utilize this resource as a host for their offspring during the period of pollen shed (*4*). Quantification of the proportion of monarch populations in each region potentially exposed to *Bt* corn pollen is difficult to ascertain. Wassenaar and Hobson (*27*), using isotope analysis of overwintering monarchs in Mexico, estimated that 50% of the monarch population originates within all or part of 15 states and one province that represent the central core of the North American Corn Belt. More than 93% of North American corn is grown in this area that extends from eastern Kansas/Nebraska to western New York. Using USDA statistics, about 28% of crop and pasture land within this area, which together constitute the monarch breeding habitat, consists of corn. Adoption of *Bt* corn across this area encompassing 50% of the monarch-breeding habitat was about 19% of the corn crop in 2000 (*28*).

Distribution of milkweeds within and around cornfields is variable across the Corn Belt. In Iowa and southern Minnesota, milkweed in cornfields represents a large proportion of the total abundance in those areas, but in Ontario and Maryland, where corn represents a smaller proportion of the landscape, milkweed in cornfields constitutes only a small proportion of its overall abundance (*4*). These data provide a variable picture, but by using data from Iowa a suitable estimate of exposure to *Bt* corn pollen by monarchs can be obtained. In addition to the fact that a significant proportion of land in Iowa is devoted to corn production, a milkweed plant in cornfields was 1.7 times more likely to receive a monarch egg than a milkweed plant in non-agricultural land (*4*). Even though milkweed densities are approximately 7 times higher in non-agricultural land than in cornfields, the proportion of monarch larvae contributed by milkweed from within cornfields is 45 times greater than that of non-agricultural land. If all of the landscape within Iowa that represents breeding habitat for monarchs is coupled with the higher per-plant egg densities on milkweeds within cornfields, the relative density of milkweeds in different habitats and the predominance of corn as a proportion of the total habitat, 56%

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of monarchs in Iowa are estimated to originate from within cornfields (*4*). This value probably differs considerably from region to region, but comparable data for other locations are not available, although the situation in Iowa likely represents the upper end of the exposure scale.

Temporal overlap of pollen shed with the presence of sensitive larvae in Iowa was 15% (*3*). An estimate of the probability of exposure ($\mathbf{P_e}$) to *Bt* corn pollen by larvae of the final monarch generation arising within Iowa can be expressed as:

$$\mathbf{P_e = l \times o \times a = 0.56 \times 0.15 \times 0.25 = 0.021,} \qquad (1)$$

or **2.1%** of the population, where $\mathbf{P_e}$ = probability of exposure, **l** = proportion of monarchs from corn, **o** = overlap of pollen shed with susceptible larval stages, and **a** = adoption rate of *Bt* corn. Because this represents an estimate of the potential exposure for Iowa, similar data would be required to estimate the probability of exposure for other locations in the Corn Belt. We do not have complete data for monarch and milkweed densities across the Corn Belt, and cannot assume that relative productiveness of crop and non-agricultural habitats is the same in other states. For Iowa, the proportion of the monarch population estimated to come from corn, 56%, was roughly similar to the proportion of the breeding habitat in Iowa that is corn. If we assume that this same relationship holds in other areas, we can use the proportion of corn grown relative to the total breeding habitat in other states as an estimate of relative monarch production.

Estimates of these three exposure factors and the estimated contribution of each state and province to approximately 50% of the eastern North American monarch population arising from the portion of the Corn Belt, as indicated by Wassenaar and Hobson (*27*), provides a broad view of potential exposure (Table 1). Our estimates of overlap of the pollen-shed period in each location with the presence of monarch larvae are based partly on the projections of the simulation model described previously and partly on our own observations. In this instance, our estimate for the exposure of monarchs in the Corn Belt states and Ontario is 1.6%. Since monarchs in the Corn Belt represents 50% of the total monarch population, the exposure for the entire monarch population would be 0.8%.

The proportion of the population of monarchs in Iowa that would be exposed to pollen levels that exceed the NOEL for each event ($\mathbf{P_t}$) can be derived from data presented in Figure 1. For event 176 pollen, monarch larvae would likely encounter pollen densities equal to or exceeding the LOEC in 90% of field situations during anthesis, while this would be true in only 0.7% or less of field situations for Bt11 and Mon810 pollen. Overall risk (**R**) is the combined probability of exposure and toxic effect or:

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Table I. Parameter estimates for probability of exposure (P_e) of monarch larvae to *Bt* corn pollen

	Parameter[1] estimates for risk of exposure		
State	l	o[2]	a
m[3]	P_e		
IA	0.560	0.15*	0.25
0.192	0.0040		
IL	0.423	0.25	0.14
0.177	0.0026		
IN	0.376	0.25	0.07
0.101	0.0007		
KS	0.119	0.15	0.26
0.038	0.0002		
KY	0.132	0.15	0.11
0.024	<0.0001	MI	0.211
0.40	0.08	0.035	0.0002
MN	0.287	0.40*	0.30
0.083	0.0028	MO	0.120
0.15	0.22	0.054	0.0002
NE	0.393	0.15	0.26
0.071	0.0011		
NY	0.135	0.40	0.11
0.003	<0.0001		
OH	0.263	0.25	0.06
0.090	0.0004		
ON	0.300	0.62*	0.20
0.020	0.0007		
PA	0.220	0.25	0.11
0.019	0.0005		
SD	0.229	0.40	0.37
0.063	0.0021		
WI	0.261	0.40	0.14
0.045	0.0007		
WV	0.026	0.15	0.11
0.005	<0.0001		
Avg. (Totals)	0.253	0.28	0.17
(1.020)	(0.0161)		

[1] **l**=proportion of monarchs from corn, **o**=overlap of pollen shed with susceptible larval stages, **a**=adoption rate of *Bt* corn.

[2] values marked with a (*) were derived from field observations in 2000 (see ref. 4).

[3] **m** represents the proportion land area of each state or province that constitutes 50% of the breeding habitat of the eastern North American monarch population.

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$$\mathbf{R = P_e \times P_t}. \quad (2)$$

If we assume that event 176 comprised 5% (**a** from equation **(1)** = 0.05) of planted corn acres in Iowa (or 20% of planted *Bt* corn acres in 2000), an extreme upper bound estimate based on historical marketing data, the risk of impact (**R**) to monarch populations exposed to effects from event 176 pollen is:

$$\mathbf{R = P_e \times P_t = 0.0042 \times 0.9 = 0.0038}, \quad (3)$$

or **0.4%** of the population.

The LOEC of pollen for all other events (Bt11 and Mon810 comprise the remaining 20% of total area planted; **a** = 0.20) equals or exceeds 0.1% of expected pollen densities, thus the proportion of the monarch population at risk of impact from effects of Cry proteins, other than event 176, in *Bt* cornfields in Iowa is:

$$\mathbf{R = P_e \times P_t = 0.0168 \times 0.007 = 0.00012}, \quad (4)$$

or **0.012%** of the population. The combined risk estimate for monarchs in Iowa is the sum of these two values, or 0.41%.

Following the same logic as above and assuming that 1) the adoption rate of *Bt* corn reached its maximum limit of 80% (**a** = 0.80) in Iowa, and 2) pollen from current and future *Bt* corn events will pose a hazard less than or equal to that established here for Bt11 and Mon810, the proportion of the monarch population in Iowa that would be at risk with market saturation is:

$$\mathbf{R = P_e \times P_t = 0.067 \times 0.007 = 0.00047}, \quad (5)$$

or **0.05%** of the Iowa population. If, instead, only event 176 hybrids were grown to the maximum extent in Iowa, 6.1% of the monarch population would be at risk. Using this format and data from Table 1, risk of exposure and toxicity from *Bt* corn can be applied to each of the states and provinces in which monarch breeding and corn production overlap.

Discussion

Previous reports (*1,21,22*) indicating the hazard of *Bt* corn pollen to monarch butterfly are inadequate to assess risk, since assigning risk can only be accomplished when both the toxicity of a potential hazard can be properly expressed and the likelihood of exposure is estimated through appropriate observations. We have utilized a comprehensive set of new data and a formalized approach to risk assessment that integrates aspects of exposure to

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characterize the risk posed to monarch from *Bt* corn pollen. Characterization of toxic effects alone indicates that the potential for hazard to monarchs is currently restricted to event 176 hybrids, which express Cry1Ab protein in pollen at a level sufficient to show measurable effects. Other events either express negligible Cry1Ab protein in corn pollen (Mon810 and Bt11) or express Cry protein of significantly less toxicity to monarch (Dbt418, Cbh351 and Tc1507 expressing Cry1Ac, Cry9c, and Cry1F proteins, respectively). Event 176 hybrids have always had a minor presence in the corn market and current plantings, which comprise <2% of corn acres, are rapidly declining.

Monarch populations share their habitat with corn ecosystems to a degree previously undocumented (*4*). Despite this, the portion of the monarch population that is potentially exposed to toxic levels of *Bt* corn pollen is negligible and declining as planting of event 176 is phased out. Because the effects portion of the risk probability equations described above ($\mathbf{P_t}$) is such a small value for the dominant corn hybrids currently planted, the sensitivity of the model to factors describing ecological exposure ($\mathbf{P_e}$) and for risk (**R**) will remain low.

Evidence supporting this risk conclusion has been collected over a wide geographic area and under a variety of conditions in both laboratory and field settings (*2-6*). Findings from studies done in multiple locations were consistent, even though methods differed from one study to another. This approach to risk characterization is consistent with accepted risk assessment procedures and shares many similarities with previous assessments over a wide range of situations describing potential risk associated with a described hazard. It is imperative that future conclusions concerning the environmental or non-target impacts of transgenic crops be based on appropriate methods of investigation and sound risk assessment procedures.

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Chapter 9

Effect of *Bacillus thuringiensis* Corn on Natural Enemies of the European Corn Borer

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Since the commercialization of transgenic corn expressing -endotoxin proteins from *Bacillus thuringiensis* Berliner genes (Bt corn), concern about the potential impact of this biotechnology-derived corn on nontarget organisms has been the focus of considerable attention. Several studies have been conducted in the laboratory and field to determine the impact of Bt corn on natural enemies (i.e., predators, parasitoids, and pathogens) of the European corn borer, *Ostrinia nubilalis*. The published literature is summarized and reviewed. Two types of experiments were conducted in Illinois to assess the impact of natural enemies on populations of first- and second-generation European corn borers. Small plot experiments were conducted in 1994 and 1995, and field-size studies were conducted in both Bt- and non-Bt cornfields during 1997 and 1998. In the small-plot trials, densities of predators in Bt and non-Bt corn plots were not significantly different during both years of the study. In the field-scale trials, corn type did not seem to affect the percentage of larvae parasitized by *Macrocentrus grandii* or the percentage of eggs and larvae infected by *Nosema pyrausta*. However, because densities of European corn borers in Bt corn were significantly lower than densities of European

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corn borers in non-Bt corn, densities of natural enemies were substantially reduced in Bt corn. Although reduced host density is predicted to coincidentally reduce parasitoid density, maintenance and effectiveness of natural enemies should be retained with establishment of non-Bt corn refuges for insect resistance management.

Introduction

The European corn borer, *Ostrinia nubilalis*, is one of the most economically important insect pests of corn in North America. Yield losses resulting from European corn borer damage and control costs exceed $1 billion in North America each year (*1*). In Illinois, European corn borers annually cause an estimated $50 million in yield losses (*2*).

The European corn borer completes two to three generations per year in Illinois. Fourth and fifth instars tunnel in stalks, causing disruption in the flow of water and nutrients in the plants. The cavities created by the borers may weaken stalks and ear shanks, resulting in lodging or dropped ears. Before the advent of Bt corn, producers attempted to control European corn borers, if the need arose, by applying insecticides before the larvae tunneled into the corn stalks. However, scouting for European corn borers, especially second generation borers, is arduous, and timing an insecticide application for maximum efficacy is difficult. Consequently, the acreage of corn in Illinois treated with insecticides for control of European corn borers has often been significantly lower than the acreage with economic infestations. In other words, many producers did nothing to manage European corn borers, similar to what has been reported throughout much of the Midwest (*3*). However, the promise of season-long control of European corn borers with transgenic corn expressing ∂-endotoxin proteins from *Bacillus thuringiensis* Berliner genes (Bt corn) (*4*) has encouraged many producers to manage this perennial pest with transgenic Bt hybrids.

As with any new technology, the use of Bt corn has raised several questions regarding potential risks. Concerns about the development of populations of European corn borer resistant to Bt have been well documented. Another primary issue regarding potential risks associated with Bt corn is the potential effect of Bt corn on predators, parasitoids, and pathogens that affect populations of European corn borer.

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The direct effect of insect resistant biotechnology-derived plants on non-target arthropods occurs by the contact or consumption of biotechnology-derived plant tissues or products of expression (e.g., pollen, nectar, sap, exudate) or possibly by consumption of pests that have fed on Bt corn. In addition, any corn hybrid that harms pest insects can alter the behavior or population numbers of non-target natural enemies of the pests. Predators and parasitoids, most of which are insects or mites, can be indirectly affected by the depletion of host/prey, changes in prey and predator/parasitoid behavior, or the consumption of the toxin that accumulated in the host/prey when they fed on the corn (*5*). However, these indirect effects also can result from other effective management tactics, such as application of chemical insecticide or biological control agents. Thus, these consequences of pest management are not unique to host plant resistance or to biotechnology-derived crops. The effects will be greater for specialist natural enemies that exclusively consume insects that damage Bt corn than for generalist predators that have a wide host range in addition to corn pests. A key point is that natural enemy populations are likely to decline to levels observed under natural, wild conditions after successful management of the pest(s) (i.e., their specific host) has occurred.

Review of the Literature

The articles reviewed herein include laboratory, small-plot, and field-scale studies that addressed the effect of Bt corn on generalist predators and specific parasitoids of the European corn borer. Several studies have focused on predators eating Bt corn products directly or on predators eating prey that had fed on Bt corn. Other studies have focused on densities of predators and/or parasitoids in Bt and non-Bt corn in small-plot or field-scale experiments. Some studies also included measurements of species diversity of natural enemies.

The predators that have been studied include flower bugs and minute pirate bugs (Hemiptera: Anthorcoridae), green lacewings (Neuroptera: Chrysopidae), ground beetles (Coleoptera: Carabidae), and lady beetles (Coleopetera: Cocinellidae). The parasitoids that have been studied include *Eriborus terebrans* (Hymenoptera: Ichneumonidae) and *Macrocentrus grandii* (Hymenoptera: Braconidae).

Over a single season, researchers in Michigan observed oviposition, predation, and parasitism of the European corn borer in 0.45-ha plots of Bt and non-Bt corn (*6*). Both kinds of plots had the same number, distribution, and size of corn borer egg masses. Levels of egg mass predation were not significantly different between the Bt corn and non-Bt corn plots. The levels of parasitism of eggs was low and was not significantly different between Bt and non-Bt corn.

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Densities of predators and parasitism of European corn borer larvae by the parasitoids *Eriborus terebrans* and *Macrocentrus grandii* were not significantly different among plots. The researchers concluded that Bt corn had no direct or indirect negative effect on populations of the predators and parasitoids measured in their study (*6*).

In small field plots in Iowa over two years, Bt corn had no detrimental effects on predators (*7*). Under laboratory conditions, Bt corn pollen caused no adverse effects when consumed directly by the insidious flower bug (*Orius insidiosus*), a green lacewing (*Chrysoperla carnea*), and the twelvespotted lady beetle (*Coleomegilla maculata*) (*7*).

In another set of studies in Iowa, densities of the parasitoid *Macrocentrus grandii* were monitored with yellow sticky traps at four locations for three summers (*8*). The primary objective of the study was to determine how different phenological growth stages of corn planted over different time intervals affected the dynamics of natural enemy populations. The numbers of adult *M. grandii* captured on traps in Bt corn were reduced by 30 to 60% compared with trap captures in non-Bt corn. The numbers of *M. grandii* captured in Bt and non-Bt corn were strongly affected by density of European corn borer hosts, the amount of stalk injury, and planting date of the corn. Planting date itself influenced host density and amount of stalk injury. Thus, low parasitoid density was associated with low European corn borer density independently of the variety of corn.

Two field studies described the effects of Bt corn on natural enemies in Italy (*9,10*). Over a 2-year period Bt corn and non-Bt cornfields had no significant differences in diversity and population abundance of the generalist predatory ground beetles (Coleoptera: Carabidae) (*9*). Similar conclusions had been drawn from an earlier, single-season study (*10*).

The predator *Orius insidiosus* was studied over a single season in the field and in several laboratory experiments (*11*). In general, densities of this predator did not differ significantly between fields of Bt and non-Bt corn. In laboratory studies, mortality nor development time of *Orius insidiosus* nymphs was affected by direct feeding on Bt corn tissue or by eating prey that had previously consumed Bt corn tissue. Similarly, another species, *Orius majusculus*, was not adversely affected by consumption of thrips that had previously eaten Bt corn (*12*).

Jasinski et al. (*13*) surveyed six fields with Bt corn, one with glyphosate-resistant corn, and five with non-biotechnology-derived corn on a weekly basis during one growing season. They observed a significantly higher density of *Orius* predators in one Bt cornfield. Numbers of *Cycloneda munda* (a lady beetle), *C. carnea* adults (a green lacewing), and predatory mites were greater in biotechnology-derived cornfields. The densities of the remaining 11 categories of beneficial insects under study were higher in fields of non-biotechnology-derived corn. More than 2000 parasitic wasps were collected in fields with

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traditional corn varities, but the difference in catch was only 5% more than in fields with biotechnology-derived corn.

Over a 2-year period, researchers in Minnesota assessed the impact of Bt sweet corn on several beneficial insects, including lady beetles, green lacewings, insidious flower bugs, and damsel bugs (Hemiptera: Nabidae) (*14*). Both fields and isolated cages of Bt corn had significantly lower densities of the lady beetle *Coleomegilla maculata*, the most dominant predator species for the duration of the study, than non-Bt corn. However, the investigators did not observe significant within-year differences between Bt and non-Bt cornfields in the overall population density of beneficial insects nor additional differences in their species diversity .

Among the most widely recognized studies that concluded a potentially negative impact of Bt corn on an insect predator were those published by researchers in Switzerland (*15,16,17*). Hilbeck et al. (*15*) performed laboratory feeding experiments with Bt corn-fed pest insects on the predaceous green lacewing *C. carnea*. Two prey species were studied, the European corn borer and *Spodoptera littoralis*. Mortality of lacewing larvae reared on Bt corn-fed prey differed depending on predator developmental stage. For example, first and second instar larvae suffered 26 and 42% mortality, respectively, when fed on Bt corn-exposed prey. Mortality of the corresponding lacewing instars fed non-Bt corn-exposed prey was 9.4 and 21%, respectively. On the other hand, mortality of third instars fed on non-Bt corn-exposed prey (6.3%) did not differ significantly from mortality of instars fed on Bt corn-exposed prey (10%).

The developmental time of lacewing larvae was not influenced by feeding on *S. littoralis* that had previously eaten Bt-corn material (*15*). Developmental time of various lacewing instars was slowed by a day or less when the larvae were given Bt corn-fed European corn borers. Hilbeck et al. (*15*) concluded that the prolonged developmental time of lacewing larvae reared on Bt corn-fed European corn borers probably occurred by a combined effect of direct toxin exposure and nutritional deficiency caused by sick prey. The applicability of this study to the field is somewhat ambiguous because the researchers did not offer a choice in prey (either different types of prey or non-exposed prey) for the lacewings, as would naturally occur for generalist predators.

Hilbeck et al. (*16*) further explored the relationship between Bt corn and *C. carnea* in diet feeding studies. Cry1Ab protein was "synthesized" in *E. coli* cells and then extracted and added to an artificial diet medium developed for the lacewing. Thus, the lacewing was directly exposed to the toxin at a single dose of 100 μg/mL. They concluded that mortality of *Bt*-exposed lacewing larvae (57%) was significantly higher than that of larvae reared on artificial diets that did not contain *Bt* toxin (30%). These artificial dietary studies suggested that some of the additional mortality in lacewing larvae that was previously observed

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by Hilbeck et al. (*15*) could have been caused by consumption of *Bt* toxin inside of the prey cadavers.

The relatively high mortality observed by Hilbeck et al. (*16*) in the control (i.e., non-Bt) treatment suggested that poor nutritional quality of the diet may have exacerbated lacewing mortality when the toxin was present. Pertinently, when green lacewings were offered a nutritious diet of insect eggs during their earliest developmental period, and then switched to an artificial diet during later development, mortality was much lower—27% for *Bt* toxin diets vs. 17% for control diets. Although these results still suggest some detrimental effect of the *Bt* toxin on green lacewings, only second instars were significantly affected.

Hilbeck et al. (*17*) confirmed the results of their earlier studies (*15,16*) by feeding *S. littoralis* prey on meridic diets containing different concentrations of Cry1Ab toxin (0, 25, 50, 100 μg/g) and then allowing lacewings to feed on the prey. Mortality of lacewing larvae fed on *Bt*-exposed *S. littoralis* was significantly greater than mortality of lacewings from the control treatment. However, this difference was observed only for lacewings exposed to *S. littoralis* that fed on the highest dose of 100 μg/g. Mortality was generally not significant at lower doses. Although this study seems more definitive, its utility in predicting the likelihood of effects under field conditions are doubtful because the tested doses do not reflect the levels of Cry1Ab in plant tissue. For example, Cry1Ab levels in event MON810, the most prevalent commercial Bt corn line, averages 10.3 μg/g in the leaves and 4.7 μg/g in the whole plant, and ranges from 0.2-0.4 μg/g in the seed (18). Considering that Hilbeck et al. (17) showed a dose-response effect for Cry1Ab and no adverse effect at a dietary toxin concentration of 25 μg/g, the actual amounts of toxin in the commercialized plant variety must be taken into consideration before concluding that lacewings will be adversely affected in the field. Nevertheless, the various studies by Hilbeck et al. emphasize the importance of examining tritrophic relationships (i.e., interactions among food source, pest, and predator) during risk assessment of biotechnology-derived plant protectants.

Other studies have lead to conclusions obverse to those of Hilbeck et al. (*15,16*). For example, the predator *Orius majusculus* was not affected by feeding on the thrips *Anaphothrips obscurus* that had fed on Bt corn (*12*). Lozzia et al. (*19*) observed no effects on *C. carnea* when it was reared on the bird cherry-oat aphid, *Rhopalosiphum padi*, an aphid feeding directly on Bt corn. ELISA (enzyme linked immunosorbent assay) analysis of the phloem sap that is consumed by aphids that feed on Bt corn failed to detect any Cry1Ab protein (*20*). The protein seems to reside in the cells, because extraction of pooled leaf samples resulted in a positive detection, indicating release from ruptured cells. Honeydew from *R. padi* that fed on Bt corn contained no measurable toxin protein, nor did the whole insect. On the other hand, the herbivorous insect *S. littoralis* and its feces contained the toxin after feeding on Bt corn. Considering

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that aphids are preyed on by lady beetles, important generalist predators in numerous agroecosystems, indirect adverse effects are unlikely to result in a tritrophic interation involving Bt corn (*20*).

Other investigators have used ELISA to measure whole body concentrations of Cry1Ab in the corn leaf aphid (*Rhopalosiphum maidis*), European corn borer, corn earworm (*Helicoverpa zea*), and black cutworm (*Agrotis ipsilon*) after feeding on diet fortified with toxin (*21*). The resulting body burdens of toxin in the tested insects were 10–100 times lower than the concentrations in the diet. When the corn leaf aphid and European corn borer fed on artificial diet with a minimum of 20 μg/g Bt toxin or higher, levels in the body were bioactive when tested in an early instar European corn borer feeding bioassay. When fed on Bt corn plants (as opposed to artificial diet), however, no significant bioactivity was found within the tissues of the prey insects when tested in the European corn borer bioassay. Thus, incorporation of toxin and subsequent bioavailability to prey is much lower when pests feed on Bt toxin incorporated into tissue than when fed on the toxin mixed into an artificial diet.

In summary, results from the aforementioned field studies do not indicate any adverse effect of Bt corn on generalist feeders (predators) nor on European corn borer specific parasitoids that have thus far been assessed. Several lab studies suggest secondary (or tritrophic) effects on predators, but these have relied on artificial diets fortified with toxin levels far above what occurs in Bt corn tissues. Aphid pests seem to pick up no toxin protein and thus pose no risk to their predators. Herbivores feeding on leaf tissue pick up substantial amounts of toxin, but one study shows that the levels are not likely to have much biological activity.

Studies on the Effect of Bt Corn on Predators and Parasitoids of European Corn Borer in Illinois

Previous studies of the impacts of Bt corn on natural enemies of European corn borer covered a relatively limited area of the Corn Belt (mainly Iowa, Minnesota, and Ohio) or were conducted only under laboratory conditions. Illinois is an important corn-growing state accounting for 16% of total U.S. production in 2002 with nearly 2.2 million acres planted to Bt corn (*22*). Thus, the effects of Bt corn on natural enemies of the European corn borer were studied specifically in Illinois from 1994 through 1998.

Population densities of predator insects were examined in small plots in 1994 and 1995. During 1997 and 1998, the impact of parasitoids and pathogens on European corn borers was examined in commercial-scale Bt and non-Bt cornfields. Based on previously reviewed studies, the experiments tested the hypothesis that generalist predator populations were not likely to be affected by the expected low densities of European corn borers in fields with Bt corn.

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However, parasitism rates were hypothesized to be low in fields of Bt corn because of significant reductions in corn borer populations.

Small-plot Studies, 1994–1995

The study site in 1994 was located near Monmouth (Warren County), IL; the study site in 1995 was located near Urbana (Champaign County), IL. Twelve treatments were included in the experiment in 1994; eight treatments were included in the experiment in 1995. The treatments during both years consisted of various combinations of Bt or non-Bt corn; no insecticide application or application of an insecticide to control first, second, or both generations of European corn borers; and manual or natural infestations of European corn borer larvae. Each treatment was four corn rows wide by 9 m long. Treatments were arranged in a randomized complete block design with six replications. One row of Bt corn was planted between adjacent plots to minimize movement of corn borer larvae from one plot to another.

The appropriate plots were manually infested with European corn borers when the corn was in the 8- to 10-leaf stage to simulate infestation by the first generation. On each of two consecutive days, approximately 100 larvae were dropped from Davis Inoculators (BioServe Products, Frenchtown, NJ) into the whorls of every plant in the center two rows of four-row plots. The manual infestation to simulate the second generation of borers was performed at anthesis (appearance of male flowers or tassels). Again, the 40 plants in each of the center two rows of the appropriate plots were manually infested with approximately 100 larvae over a 2-day period. The larvae were applied to the leaf axils in the ear zone (ear leaf, three leaves above the ear, and three leaves below the ear).

Evaluations of injury caused by European corn borers, assessment of densities of European corn borer larvae, and measurements of corn yield were conducted. However, for the purposes of discussion, only the procedures used to assess populations of predator insects are provided.

To determine whether densities of insect predators differed among treatments, all plots were sampled from the time corn plants were at the 8- to 10-leaf growth stage until harvest. The plots were monitored weekly in 1994 and bimonthly in 1995. Numbers of lady beetles, damsel bugs, insidious flower bugs, bigeyed bugs (Hemiptera: Lygaeidae), and green lacewings were recorded. In both 1994 and 1995, predators were counted on five plants in each of the center two rows of each plot, for a total of 10 plants per plot. The data collected from the various treatments were statistically compared with Chi-square analysis.

Overall, the counts of beneficial insects per plot were low (12 to 15 per 30 plants). Often no beneficial insects were found in the plots, possibly because walking through the plots disturbed the insects and they either moved out of the

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plots or were hidden from observation. However, when beneficial insects were present, they seemed to be evenly distributed among the plots. Anaysis of the data revealed no significant differences in densities of beneficial insects among the plots. In 1995, a large population of *O. insidiosus* occurred in the plot area. Although a significant difference in the number of *O. insidiosus* occurred over time, no significant differences among plots were evident.

Bt corn did not have a negative impact on poulations of predators during the two years of the small-plot studies. However, densities of predators in small plots may not reflect population dynamics of natural enemies in cornfields. Most insect predators and parasitoids are mobile, so they likely moved freely from plot to plot, which would occur in any small-scale experiment. Thus, a more realistic assessment of the potential impact of Bt corn on natural enemies of European corn borers was conducted in field-sized experiments.

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Field-Scale Studies, 1997–1998

Studies were conducted during 1997 and 1998 in two no-till commercial-scale fields of Bt corn and in two no-till commercial-scale fields of non-Bt corn. The fields were located near Auburn (Sangamon County), IL. All fields were sampled weekly from June through August for life stages of European corn borers, including egg masses, and the effects of the parasitoids *Macrocentrus grandii* and *Trichogramma* sp. (Hymenoptera: Trichgrammatidae) and the pathogens *Nosema pyrausta* (Microsporidia: Nosematidae) and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes).

In general, there were no significant differences between densities of European corn borer egg masses in Bt and non-Bt cornfields, a finding similar to that reported from small plots by Orr and Landis (*6*). However, because of the efficacy of Bt corn against European corn borer larvae, there were significant differences in densities of larvae in Bt and non-Bt corn during both years.

Corn type did not seem to affect the percentage of European corn borer eggs parasitized by *Trichogramma*, the percentage of larvae (except second-generation larvae in 1998) parasitized by *M. grandii*, and the percentage of European corn borers infected by *N. pyrausta* and *B. bassiana*. The percentage of larvae parasitized by *M. grandii* was significantly higher in second-generation European corn borers in non-Bt corn in 1998; no parasitoids were obtained that year from larvae collected from Bt cornfields. As hypothesized, densities of natural enemies were substantially reduced in the fields with Bt corn in association with reduced populations of European corn borer.

A reduction of parasitoids in Bt cornfields should not be interpreted as having negative consequences on the maintenance and effectiveness of natural enemies with widespread planting of Bt corn. Non-Bt corn refuges are required to be planted as part of insect resistance management plans (*18*). Thus, if these

blocks of corn are not treated with insecticides, parasitoid abundance should vary in accordance with the corn borer populations. Predators are unlikely to be affected because they are generalists and have many hosts in addition to corn borers.

Conclusions

The results from numerous published field studies suggest that Bt corn has no direct negative impacts on populations of several natural enemies of European corn borers. Results from the studies in Illinois support this general conclusion. However, the interactions of crops, pests, natural enemies, and environment are complex, so many factors must be considered when recommendations are developed for deployment of Bt corn and non-Bt corn. For example, Obrycki et al. (*23*) have speculated that if European corn borer densities are significantly suppressed by the use of Bt corn, the expected consequent reduction in abundance of specific parasitoids might hasten the development of resistant pest populations. The reduction in efficiency of the parasitoids as a mortality factor for European corn borer migh allow the rare Bt resistant individuals to flourish. While such concerns should be alleviated by required Bt resistance management refuges, the possibility in essence has already been tested with the long time release of corn varieties bred for resistance to first generation European corn borer. Thus far, no reports have surfaced to suggest that host plant resistance based on traditional breeding has resulted in resistant European corn borers, nor have effects on parasitoids been noted.

Another concern expressed by Obrycki et al (*23*) was that negative impacts of Bt corn on natural enemies raised the possibility that overuse of transgenic corn could lead to the types of resurgence and secondary-pest outbreaks that are associated with misuse of synthetic broad-specturm insecticides. However, evidence presented on interactions between natural enemies and transgenic insecticidal corn (Bt corn) in the published literature showed that Bt, for the most part, did not have a negative impact on natural enemies. For example, published information assembled by Obrycki et al. (23) showed 13 examples for which Bt corn had no effect on natural enemies, three examples for which Bt corn had a negative effect on natural enemies, and two examples for which Bt corn had a positive effect on natural enemies. Thus far, most studies do not suggest any adverse impact of Bt corn on European corn borer natural enemies.

The studies from Illinois discussed herein and at least one study in Iowa (*8*) have revealed that densities of the parasitoid *Macrocentrus grandii* could be reduced in Bt cornfields, primarily as a consequence of the reduction of the numbers of European corn borers in Bt corn. *Macrocentrus grandii* is considered to be one of the three most important insect species that parasitize European corn borers in North America and Europe (*24*). Therefore, a reduction

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in numbers of *M. grandii* in Bt corn could ultimately affect population dynamics of European corn borers. Such effects may also occur when borers are controlled by insecticide sprays, but insecticides are also likely to kill the generalist predators. Predators are unlikely to be affected by the currently registered Bt corn lines owing to foliar toxin levels that are low enough to prevent a toxic dose from being transferred during tritrophic interactions. Such a conclusion is warranted based on laboratory dose-response studies with green lacewings (*17*).

Conservation of natural enemies in the corn ecosystem is important for suppressing insect pest populations. The refuges of non-Bt corn that are recommended for resistance management also can be used for conservation of natural enemies. If non-Bt corn refuges are planted nearby Bt corn, as recommended, the impact on natural enemies of European corn borers, and therefore other pests, should be minimal.

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Chapter 10

Allergy Assessment for Food Biotechnology

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Establishing the safety of foods derived from GM crops requires a multidisplinary approach with methods adapted from the biochemical, nutritional, toxicological and immunological sciences. The core principle of the process has been articulated as *substantial equivalence*, which is a comparative evaluation of a large number of analytes from transgenic and non-transgenic crop varieties. For allergy, it is essential to evaluate the introduced protein and, for highly allergenic crops, it may also be necessary to evaluate potential changes in endogenous allergens. The prevalence of food allergies appears to be on the rise, particularly in developed countries. Since no cure is available for those afflicted with food allergy, disease management is achieved by avoidence of the offending food. As a result, significant weight in the assessment is given to the need for prevention, which in the context of safety assessment, means, among other approaches, reducing the likelihood of transferring offending allergens from one food to another. Genetic engineering of food crops should have little practical consequence for the occurrence, frequency, and natural history of food allergy if this evaluation is robust. Essential aspects of allergy assessment are discussed in this chapter.

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Introduction

World population is expected to increase by 2.5 billion people in the next 25 years. Concomitantly, the food requirements for this growing population are expected to double by the year 2025. In contrast, there has been a decline in the annual rate of increase in cereal yield such that the annual rate of yield increase is below the rate of population increase. In order to feed this growing population, crop yield will have to be increased and some of the increase in yield will be due to genetic engineering of foods.

A consumer survey regarding the prevalence of adverse reactions to foods indicated 30% of the people interviewed felt that they or some family member had an allergy to a food product (1). This survey also found that 22% avoided particular foods on the mere possibility that the food may contain an allergen. In reality, food allergic reactions affect only 6-8% of children and 2-2.5% of the adult population (2,3) and are elicited by a small subset of the foods we consume. The most common food allergies known to affect children are IgE-mediated reactions to cow's milk, eggs, peanuts, soybeans, wheat, fish, crustacean shellfish and tree nuts. Approximately 80% of all reported food allergies in children are due to peanuts, milk, or eggs. While most childhood food allergies are outgrown, allergies to peanuts, tree nuts, and fish are rarely resolved. In adults the most common food allergies are to peanuts, tree nuts, fish, and crustacean shellfish. Allergic reactions are typically elicited by relatively few proteins that are found in abundance in commonly eaten foods.

Numerous commodity crops have been genetically modified (GM) by the introduction of specific genes encoding proteins that provide protection from insect pests or tolerance to herbicides. These GM crops are useful for reducing the need for insecticides and allowing reduced tillage farming and all of them have been approved for commercial production in many countries (4). The approval process that allows each of these products to be used in commercial production involves safety assessments that are designed to protect the health of the human and/or animal consumer, and the environment. The safety assessment of GM crops is quite extensive, usually involving comparative studies of the modified crop with current commercial varieties with regard to composition, nutrition, and environmental impact. An essential part of the safety assessment

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similarity to structural and biochemical characteristics of known allergens. This assessment involves a series of investigations including evaluation of the source of the transgenic protein, determination of the degree of amino acid sequence homology with known allergens, reactivity by immunoassay using the sera of individuals with known allergies to the source material, degree of pepsin resistance, and in some cases reactivity by immunoassay using the sera of individuals with known allergies from related materials, and when available, immunogenicity in suitable animal models predictive for allergenic potential. For crops which contain significant levels of endogenous allergens, such as soybeans, it is also nessecary to show that that there is a limited impact on endogenous allergens quantitatively and qualitatively, also by immunoassay.

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Analyzing the Source of Introduced Genes

The source of the introduced gene is the first variable to consider in the allergy assessment process. If a gene transferred into a food crop is obtained from a source known to be allergenic, data should be generated to prove that the gene does not encode an allergen. The U.S. Food and Drug Administration (FDA) recognizes this need and realizes that such risks to consumers can be avoided (5). The use of labels that clearly indicate the presence of ingredients that may cause harmful effects, such as allergies, gives consumers the opportunity to avoid these foods or food ingredients. For example, to assist people who suffer from celiac disease, the FDA has determined that products containing gluten should be identified as to the source – i.e., wheat versus corn gluten (wheat gluten cannot be safely consumed by these patients, unlike corn gluten). In the case of food allergy, voluntary advisory labeling already occurs for certain foods that do not ordinarily contain peanuts, but that may come into contact with peanuts during processing or preparation. This type of labeling provides protection for peanut allergy sufferers and helps prevent accidental and unwanted exposure. The FDA has also stated that, if known allergens are genetically engineered into food crops, the resulting foods must be labeled to disclose the source of the introduced genes (8). Moreover, proteins derived from known allergenic sources should be treated as allergens until demonstrated otherwise. The methodology to assess whether the transferred protein is allergenic is described below.

Amino Acid Sequence Comparisons to Known Allergens

The proteins introduced into all genetically engineered plants that have been put into commerce in the U.S. have been screened by comparing their amino acid sequence to those of known allergens and gliadins as one of many assessments performed to evaluate product safety (9,10). Additionally, the amino acid sequence of the introduced protein is screened against all known proteins in publicly available sequence databases to identify proteins that could have other potential safety concerns. The extent of sequence similarities between the introduced protein and database sequences of allergens, gliadins, and other proteins can be efficiently assessed using the FASTA sequence alignment tool (11). Although the FASTA program directly compares amino acid sequences (*i.e.*, primary protein structure), the alignment data may be used to infer higher order structure (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout their entire length are often homologous. Homologous proteins share secondary structure and common three-dimensional folds (12). Homologous proteins are more likely to share allergenic cross-reactive conformational and linear epitopes than unrelated proteins; however, the degree of similarity between homologues varies widely. Aalberse (13) has noted that proteins sharing less than 50% identity over their entire length are unlikely to be cross-reactive, and only when they share more than 70% identity does cross-reactivity become common. A recent FAO/WHO scientific panel recommended using an overall structural homology of greater than 35% as a conservative basis for a match with known allergens (7).

There is some concern that the FASTA search might miss short regions within a protein that are identical or highly similar in sequence to an existing allergen and have the potential to elicit clinical symptoms (IgE-binding epitopes) or to sensitize susceptible individuals (T-cell epitopes). Since IgE-binding epitopes and T-cell epitopes have only been identified for a few allergens, it is not possible at this time to construct a comprehensive epitope database for a more accurate search. Further, most of the "epitopes" that have been identified are known through *in vitro* mapping studies without regard to antibody affinity and using sera from only a few allergic individuals. While some IgE epitopes may be as short as five amino acids (14,15), the majority of characterized IgE-linear epitopes are eight amino acids or longer (16-18) and T-cell epitopes are typically at least 8 amino acids or longer. Although many reports have demonstrated IgE-binding, few have tested the affinity (avidity) of the binding or the allergic significance of the *in vitro* binding, and it is clear from some reports that high affinity binding requires eight or more amino acids (14,19).

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In the absence of a complete description of IgE epitopes for all known allergens, a theoretical database of all potential epitopes for these same allergens can be effectively screened by scanning all overlapping peptides (in this case eight or more amino acids in length) of all the allergens of the database and comparing them in pair-wise fashion to all same-size potential peptides of the test protein using computer software or scanning manually. This can be viewed as a highly conservative and all inclusive approach as most of the theoretical peptides compared with the query sequence do not represent *bona fide* epitopes. A recent FAO/WHO scientific panel recommended using a six amino acid window for this type of analysis (7). However, Hileman et al. (20) demonstrated that an amino acid window size of less than eight residues resulted in the identification of many irrelevant sequences. Therefore, the use of an eight amino acid window represents a compromise to identify most of the potentially cross-reactive single IgE epitopes, while reducing the probability of identifying a large number of irrelevant similarities that would be identified using a window smaller than eight amino acids. It should also be recognized that two IgE-binding epitopes on the same molecule are required to cross-link high affinity IgE receptors on mast cells and induce an intracellular signal. If sufficient numbers of receptors are stimulated, the mast cell will degranulate, releasing histamine and leukotrienes. Therefore, a single match in this analysis may or may not be clinically significant.

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In vitro and *In vivo* IgE Immunoassays

If the gene being introduced is from a known allergenic source or if the bioinformatic search of the gene product results in significant sequence homology with a known allergen, *in vitro* and *in vivo* assays are employed to determine if the target protein is likely to bind bioactive IgE.

Specific and Targeted In vitro Immunoassays

In vitro assays such as radioallergosorbent tests (RAST; 21,22), enzyme-linked immunosorbent assays (ELISA; 23), or immunoblotting assays should be undertaken to determine if an allergen has been transferred to the target plant. These assays use IgE fractions of serum from appropriately sensitized individuals who are allergic to the food from which the transferred gene was derived. Serum donors should meet clinically relevant criteria, including a convincing history (24) or positive responses in double-blind, placebo controlled food challenges (21,25). *In vitro* assays should be validated with respect to their specificity, sensitivity, and robustness in identifying the target protein. A recent

FAO/WHO scientific panel (7) has recommended that in addition to using serum IgE from individuals who are allergic to the food from which the transferred gene was derived, serum IgE from patients allergic to plants in the same botanical family also be used in these assays. However, since *in vitro* IgE cross-reactivity can occur without clinical symptoms being elicited, a postive result in such an assay may be difficult to interpret. Positive results from one or more of these *in vitro* assays indicates that a potential allergen or a cross-reactive allergen has been transferred and would require that any food with the transferred gene be labeled as containing a gene from that source.

In vivo Assays

For transgenic proteins from allergenic sources or with significant sequence homology with known allergens, further evaluation is required to determine if the introduced protein could precipitate IgE-mediated reactions. *In vivo* skin prick testing may be required for some proteins. Skin prick testing is an excellent negative predictor of allergenicity but is only 50-60% predictive of clinical symptoms if a positive result is obtained and wheal diameter is not taken into account (26). The best *in vivo* test of allergenicity is the double-blind-placebo-controlled-food challenge (DBPCFC). This procedure involves testing with allergic and nonallergic patients under controlled clinical conditions. Patients who are known to be allergic to proteins from the source would be tested directly for hypersensitivity to food containing the protein encoded by the gene from the allergenic source. Food from the parental variety would be used as a negative control. The ethical considerations for this type of assessment would include factors such as the likelihood of inducing anaphylactic shock in test subjects, potential value in testing subjects, availability of appropriate safety precautions, and approval of local institutional review boards. If sensitive patients underwent a reaction in these tests, food derived from crops containing the protein would require labeling.

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Assessment of Pepsin Digestibility

The pepsin digestibility assay was conceived as a means to determine the relative stability of a protein to the extremes of pH and pepsin protease encountered in the mammalian stomach and was originally developed and utilized as a method to assess amino acid bioavailability (27-29). The logic behind this test was that proteins that are nutritionally desirable tend to be rapidly digested and have greater bioavailability of amino acids than stable proteins. In addition, proteins that are highly digestible would be expected to

have less opportunity to exert adverse health effects when consumed. Standardization of the assay conditions (i.e. pepsin concentration, pH, temperature, etc.) has been described in the U.S. Pharmacopia (30) and is sometimes referred to as simulated gastric fluid (SGF). The assay was not meant to precisely mimic the fate of proteins in *in vivo* conditions, but rather to evaluate the susceptibility of the protein to digestion under fixed conditions *in vitro*. The purpose is to provide information that, in conjunction with other evidence, would be useful in predicting whether a dietary protein may become a food allergen. Therefore, the relationship of the resistance to digestion by pepsin and the likelihood that a dietary protein is an allergen was identified and subsequently recommended by the U.S. FDA, EPA, and USDA in 1994 (8) as a means of aiding the assessment of proteins added to commodity crops through biotechnology.

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The digestive stability of the major allergens found in the most common allergenic foods were the first to be studied (31). The stability of some of the major allergens of peanut, soybean, egg, and milk relative to the stability of common non-allergenic food proteins were determined in the standard pepsin digestion assay (31). Under the conditions described for SGF in this study, all food allergens were more resistant to pepsin hydrolysis than were common plant proteins. For example, the Ara h 2 allergen of peanut was stable for at least 60 minutes in the pepsin digestion assay while other non-allergen plant proteins such as rubisco (spinach leaf) or acid phosphatase (potato) were digested in less than 15 seconds. However, not all allergens from the most common allergenic foods were stable in the pepsin digestion assay for 60 minutes. Stability of the whole protein or fragments from the allergens tested ranged from 8 minutes to 60 minutes whereas all of the non-allergenic plant proteins tested did not survive in the pepsin digestion assay for more than 15 seconds.

Since this initial report, there have been numerous studies repeating the pepsin digestion assay on these major food allergens (32). In general, the original findings that these allergens were stable to pepsin digestion relative to non-allergenic proteins were confirmed but the length of time that either the whole protein or fragments of the allergen were stable did not always agree. The most likely explanation for this quantitative difference is due to subtle changes in the pepsin digestibility assay or in the method by which the proteins of interest were detected. For example, changes in enzyme concentration, pH, protein purity, and method of detection could have large effects on the interpretation of any *in vitro* assay. For this reason, the International Life Sciences Institute (ILSI) has proposed a standardization process for the assay that will attempt to assess these variables so that results from different laboratories can be directly compared. Federal, academic and industry labs from Europe, North America

and Japan will participate in this test where pH (1.2/2.0), pepsin concentration, allergen purity and method of detection have all been standardized (33).

Animal Models for Predicting Allergenicity

There has been considerable interest in the development of animal models that would permit a more direct evaluation of the sensitizing potential of novel proteins. In this context, attention has focused on the production of IgE in response to the novel protein and a wide variety of models are being developed for this purpose including rodents (34-36), dogs (37) and swine (38). A variety of variables are being tested in the development of each model. Some of the variables being tested are route of sensitization, dose, use of adjuvant, age of organism, diet and genetics. Unfortunately, there are no validated models currently available for assessing the allergenic potential of specific proteins in naïve subjects. This is in part due to the extremely complex nature of the immune response to foods and proteins and in part due to the fact that most of the animal models of food allergy were originally developed to understand the mechanisms of allergenicity rather than assessing the allergenic potential of novel proteins. While some progress is being made in select models (38-40) there still remains much work to be done before there is confidence that any one model will provide positive predictive value with regard to protein allergenicity.

Evaluation of Endogenous Allergens

From the perspective of human health risk, it is possible that substantive change in the allergenicity of allergenic foods leading to increased incidence or severity of food allergy could be affected by genetic modification and therefore should probably be evaluated and considered in the safety assessment. To date, evaluations of endogenous allergens in genetically modified foods have typically been performed for crops which fall into the top eight "commonly" allergenic food groups. Experimentally, these evaluations involve *in vitro* IgE immunoassays including immunoblotting, ELISA, ELISA inhibition, or a combination of these techniques. Examples of such approaches include the initial evaluation of Roundup Ready® soybeans by immunobloting (41) and more recently using both immunoblotting and ELISA techniques (42). Both studies concluded that there were no meaningful differences between genetically modified and traditional soybeans.

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Conclusions and Future Considerations

The allergy assessment testing strategy, as it is presently formulated, is a tiered, hazard identification approach that utilizes currently available scientific data regarding allergens and the allergic response. While a hazard assessment approach has generally worked to ensure the safety of the current wave of pest resistant and herbicide tolerant crops, it may not be adequate to use in the assessment of the next generation of nutritionally enhanced GM foods, because the level of exposure to the novel protein may be much higher. It is evident that the testing strategy will need to be integrated into a risk assessment model where risk is defined as a function of the level of the hazard and the level of exposure to the hazard. This strategy consists of four steps: hazard assessment, dose-response evaluation, exposure assessment, and risk characterization (43). To apply risk assessment principles to the issue of the allergenicity of proteins and GM crops new scientific data must be collected for each step in this process. In fact, this has already begun. For example, the issue of dose-response evaluation is beginning to be addressed by a variety of investigators exploring threshold doses for different foods in allergic patients (44). The issue of exposure assessment consists of three parts: the abundance of the protein in the food, the stability of the protein in the GI tract and the amount of the GM crop consumed in the diet. Efforts to collect data on all three parts of this important step in the risk assessment paradigm have already begun.

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Chapter 11

Role of Compositional Analyses in the Evaluation of Substantial Equivalence for Biotechnology Crops

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The concept of substantial equivalence was developed as a scientific and practical approach for the safety assessment of biotechnology corps. The analysis of the nutritional composition of a biotech crop and a comparison to conventional crop counterparts is a key step in this process. The data for comparison should be obtained from samples produced in controlled field trials and then analyzed using validated methods and appropriate statistical techniques. It is also important to account for natural variability in composition due to genetic and environmental factors. The evaluation of the composition of Roundup Ready® soybeans and corn by these techniques has demonstrated that these biotech crops are as nutritious and as safe as conventional soybeans and corn grown today.

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Herbicide tolerance has been introduced, through genetic modification, into a number of crops. Glyphosate, which is the active ingredient in the herbicide, Roundup®, is one of the most widely used herbicides in the world. Since 1996, glyphosate-tolerant or Roundup Ready® crop varieties have been developed and commercialized for soybean (*Glycine max*) (1,2), canola (*Brassica napus*), cotton (*Gossypium hirsutum*) (3) and corn (*Zea mays* L.) (4).

The safety assessment of foods or feeds derived from crops improved through biotechnology accounts for the two major sources of potential health consequences: 1) direct effects due to the activity and presence of the introduced trait (most often a protein) and 2) indirect effects due to the introduction of the genetic material and the characteristics of the resulting food or feed crop plant (5). Table I summaries the types of studies that have been conducted in the safety assessment of biotech crops and emphasizes the concept that no single experiment would be expected to give a conclusive answer, but rather the weight of all evidence needs to be considered in reaching a conclusion regarding the safety of a food or feed derived from a crop improved through biotechnology.

Table I. Evaluation of the Food and Feed Safety for Biotech Crops

Test Substance	*Study/Experimental Parameter*
Gene	Source(s)
	Molecular characterization
	Insert/copy number/gene integrity
Protein	History of safe consumption
	Function/specificity/mode-of-action
	Expression levels
	Toxicology/allergenicity
Whole Crop	Agronomic performance
	Composition
	Livestock feed performance
	Rat sub-chronic toxicity

The safety assessment of the crop has the advantage that comparisons can be made to traditional crops, in a process referred to as substantial equivalence, WHO (6, 7); FAO (8); OECD (9, 10, 11). An evaluation of the composition and nutritional profile of the biotechnology crop and a comparison to its conventional crop counterpart is a key step in the safety assessment process.

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In this chapter two crops, Roundup Ready soybeans and Roundup Ready corn, are used as examples to illustrate the role of compositional analyses in the evaluation of substantial equivalence.

Materials and Methods

Roundup Ready soybean line 40-3-2 was produced by the stable insertion of a gene that encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) (12). Roundup Ready corn event NK603 was produced by the stable insertion of two gene cassettes, that express CP4 EPSPS proteins (13). 5- Enolpyruvylshikimate-3-phoshate synthase is present in plants, bacteria and fungi, but not in animals, as a component of the shikimate pathway of aromatic amino acid biosynthesis. In soybeans and corn, the presence of CP4 EPSPS, confers glyphosate tolerance to the plants while meeting the needs for the production of aromatic amino acids.

As shown in Table II, there are four major steps in the evaluation of compositional equivalence of a biotech crop.

Table II. Steps in the Analysis of Composition for a Biotech Crop

Step	*Area of Investigation*
1.	Field Trials
	Production plan
	Multiple locations
	Replicated test, control and reference plots
2.	Identity of test, control and reference samples
	Southern blot assay
	Polymerase chain reaction assay
	Chain-of-custody records
3.	Analysis of composition
	Protocol (GLP)
	Validated methods
4.	Statistical Evaluation

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Those four steps are – 1) conducting field trials, 2) assessing the identity of test, control and reference samples, 3) analysis of composition and 4) statistical evaluation of results. Field trials typically involve planting the biotech crop in at least four different geographic locations that span the regions where the crop would be grown commercially. The crop should be grown under commercial conditions with herbicide applied to a herbicide-tolerant crop. The biotech crop is planted in a randomized complete block design with an isogenic or near isogenic relative that does not contain the biotechnology trait. In most cases the transgenic and non-transgenic crops are replicated using three to four replications or blocks. In addition conventional commercial varieties of the crop may be included in the field trail to provide samples for comparative analysis. These conventional commercial varieties can be particularly important if there is little data in the literature on the range of values for key biochemical components since they provide an estimate of the variability of the level of individual nutrients.

Forage and grain samples from the field trial are harvested, labeled, frozen, processed and shipped to the laboratory for analysis. The identity of the biotech crop is established by genetic analysis or analysis of the gene product (protein) by enzyme-linked immunosorbent assay (ELISA). The genetic assay may be an event specific Southern blot or polymerase chain reaction (PCR) assay. The assays establish the identity of the materials by demonstrating the presence or absence of the biotechnology trait in a grain or forage sample. Chain-of-custody records can also be used to document the identity of individual samples.

Compositional analysis of the forage and grain samples usually includes the analysis of proximates (total protein, total fat, ash, moisture and carbohydrate by calculation), fiber, amnio acids, fatty acids, vitamins, minerals, anti-nutrients and additional metabolites of interest. The biochemical components analyzed for Roundup Ready corn NK603 are shown in Table III (13).

The methods used for these analyses are typically methods validated by professional societies such as the Association of Official Analytical Chemists (AOAC), the American Association of Cereal Chemists (AACC) or the American Oil Chemists Society (AOCS). The samples from the biotech crop, its isogenic or near isogenic relative and conventional varieties are analyzed using the same methods in a randomized order to prevent bias. Additional documentation of the performance of the equipment and methods as well as the collection and calculation of the results is provided by the guidelines of Good Laboratory Practices (GLP). The statistical analysis of compositional data is conducted following the conversion of individual analyte values from fresh

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weight to a dry weight basis. Frequently a mixed model analysis of variance is used for each replicated site and for a combination of all sites for the comparison of the biotech crop to its isogenic or near isogenic control. It is common to use a statistical program such as SAS (SAS Institute, Cary, North Carolina) to perform the analyses.

Table III. Biochemical Components Measured in Roundup Ready Corn (NK603)

Forage and Grain	*Grain*
Protein	Amino acid profile
Fat	Fatty acid profile
Ash	Phytic acid
Moisture	Ferulic acid
Carbohydrate by calculation	p-Coumaric acid
Neutral detergent fiber	2-Furaldehyde
Acid detergent fiber	Raffinose
	Vitamin E
	Minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, zinc)

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Results of Compositional Analysis

Proximate Analyses in Roundup Ready Soybeans

Soybeans are an important source of protein and oil for the food and feed industries. Therefore, the levels of total protein and oil in Roundup Ready soybeans and its near isogenic control were evaluated and the results were compared. Table IV contains the data for the analysis of proximates (protein, oil, ash, moisture and carbohydrate by calculation) for Roundup Ready soybeans (40-3-2) treated with glyphosate and control soybeans (A5403) (2).

Table IV. Proximate Analysis of Roundup Ready Soybeans from 1993 Field Trials

Component	*Roundup Ready 40-3-2*		*Control A5403*	
	Mean	*Range*	*Mean*	*Range*
Protein (% dw)	41.4	40.39-4.32	41.43	39.35-44.14
Ash (% dw)	5.31	5.01-5.94	5.35	5.04-5.81
Moisture (% fw)	5.73	5.18-6.19	5.74	5.32-6.20
Fat (% dw)	19.89	18.67-20.57	20.53	19.01-22.17
Carbohydrates (% dw)	33.38	31.57-35.08	32.67	27.86-35.32

Statistical analysis of the data indicated that there were no significant differences at the 95% confidence level between the transgenic and control varieties.

Additional Nutritional Evaluation for Roundup Ready Soybeans

The mean and range of values for the amino acid analysis of Roundup Ready soybeans (40-3-2) and its non-transgenic control line (A5403) grown in the U.S. in 1993 are shown in Table V (2). A statistical comparison of the two sets of data indicated that there were no statistically significant differences between Roundup Ready soybeans (40-3-2) and the control line. This observation was particularly important for the aromatic amino acids phenylalanine, tyrosine and tryptophan since 5-enolpyruvylshikimate synthase (EPSPS) is known to be an essential enzyme in the biosynthesis of aromatic amino acids, and the Roundup Ready soybean plants in this trial were treated with the herbicide glyphosate which is known to inhibit wild-type EPSPS. These results indicate that the glyphosate tolerant enzyme (CP4 EPSPS) is capable of supplying the intermediates in the shikimatic acid pathway at equivalent levels, even in the presence of glyphosate. Additional analyses of isoflavones (1, 2), fatty acids, trypsin inhibitor and lectin (1) indicated that there were no biologically significant differences between Roundup Ready soybeans and its control. Roundup Ready soybeans were also shown to have a equivalent feed value in rats, poultry, catfish and dairy cattle (14).

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Table V. Amino Acid Analysis of Roundup Ready and Parental Control Soybeans from 1993 U.S. Field Trials (g/100g dry weight)

Amino Acid	*Roundup Ready 40-3-2*		*Control A5403*	
	Mean	*Range*	*Mean*	*Range*
Aspartic Acid	4.51	4.21-4.75	4.50	4.30-4.59
Threonine	1.57	1.52-1.63	1.53	1.54-1.60
Serine	2.03	1.92-2.11	2.03	1.95-2.06
Glutamic Acid	7.44	6.84-7.97	7.48	7.06-7.81
Proline	2.06	1.91-2.16	2.07	1.97-2.16
Glycine	1.71	1.61-1.78	1.73	1.65-1.83
Alanine	1.72	1.67-1.76	1.73	1.66-1.79
Valine	1.93	1.83-2.00	1.94	1.87-1.97
Isoleucine	1.82	1.71-1.91	1.83	1.75-1.88
Leucine	3.06	2.90-3.19	3.09	2.96-3.16
Tyrosine	1.38	1.32-1.44	1.39	1.34-1.41
Phenylalanine	1.98	1.86-2.08	2.01	1.92-2.06
Histidine	1.10	1.05-1.15	1.10	1.07-1.12
Lysine	2.64	2.53-2.76	2.63	2.53-2.69
Arginine	2.89	2.64-3.09	2.88	2.70-2.97
Cysteine	0.59	0.54-0.60	0.57	0.50-0.61
Methionine	0.54	0.51-0.55	0.54	0.48-0.57
Tryptophan	0.49	0.47-0.53	0.49	0.48-0.50

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Proximate Analyses in Roundup Ready Corn Event NK603

Over 90% of the corn raised in developed countries is used in animal feed (15) therefore an analysis of the nutritionally important components in Roundup Ready corn is extremely important to evaluate the nutritive value of this product for livestock. In Table VI the levels of proximates, acid detergent fiber (ADF) and neutral detergent fiber (NDF) are presented for Roundup Ready corn event NK603 and its non-transgenic control obtained from field trials in 1998 (13).

Table VI. Proximate Analysis of Roundup Ready (NK603) and a Control Corn Grain Collected from 1998 Field Trials

Component	*Roundup Ready NK603*		*Control*	
	Mean	*Range*	*Mean*	*Range*
Protein (% dw)	12.20	10.30-14.77	12.60	11.02-14.84
Ash (% dw)	1.45	1.28-1.62	1.49	1.32-1.75
Moisture (% fw)	11.13	9.01-13.30	11.78	8.56-14.80
Fat (% dw)	3.61	2.92-3.94	3.67	2.88-4.13
Carbohydrates (%dw)	82.76	80.71-84.33	82.29	80.23-83.70
ADF (% dw)	3.72	3.14-5.17	3.60	2.79-4.28
NDF (% dw)	10.06	7.89-12.53	10.00	8.25-15.42

No significant differences between NK603 and its non-transgenic control were observed, indicating that Roundup Ready corn is as nutritious as conventional corn.

Additional Nutritional Evaluation of Roundup Ready Corn Event NK603

As indicated in Table III, a comprehensive evaluation of the nutritional status of corn event NK603 was conducted including the analysis of fatty acids, amino acids, minerals, anti-nutrients (phytic acid and raffinose), vitamin E and secondary metabolites (13). Listed below, in Table VII, are the results for the analysis of fatty acids for NK603. All of the fatty acids measured in grain samples from this 1999 field trial in E.U. were not statistically different at the $p<0.05$ level except for the minor fatty acid, 20:0 arachidic acid (13). A comparison of the range of values for NK603 to the tolerance interval for the conventional commercial varieties demonstrates that the levels of arachidic acid are well within the population of the conventional varieties grown at the same locations. This indicates that small statistically significant differences are unlikely to be of biological relevance. Similar results were obtained for analyses of other biochemical components indicating the Roundup Ready corn event NK603 is as safe and nutritious as conventional corn. Subsequent feeding performance studies in livestock including broiler chickens (16) and swine (17)

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Table VII. Fatty Acid Analysis of Corn Grain from Roundup Ready Hybrid NK603, Control and Reference Commercial Hybrids from 1999 Field Trials (% of Total Fatty Acids)

Fatty Acid	*Roundup Ready NK603 Mean (Range)*	*Control Mean (Range)*	*Commercial Reference Tol. Interval*[a]
Arachidic acid (20:0)	0.36[b] (0.34-0.39)	0.35 (0.33-0.37)	0.17, 0.64
Behenic acid (22:0)	0.16 (0.12-0.20)	0.18 (0.15-0.19)	0.093, 0.24
Eicosenoic acid (20:1)	0.30 (0.28-0.34)	0.29 (0.28-0.31)	0.21, 0.42
Linoleic (18:2)	63.73 (61.94-65.25)	63.15 (61.63-64.04)	44.59, 73.50
Linolenic (18:3)	1.02 (0.97-1.05)	1.09 (1.05-1.12)	0.54, 1.72
Oleic (18:1)	23.80 (22.82-24.95)	24.20 (23.52-25.56)	12.65, 39.86
Palmitic (16:0)	8.90 (8.47-9.36)	9.00 (8.89-9.13)	7.35, 14.72
Stearic (18:0)	1.73 (1.59-1.88)	1.74 (1.67-1.81)	1.02, 2.27

[a] Tolerance interval is specified to contain 99% of the commercial line population with a confidence level of 95%.
[b] Statistically different from the control at the 5% level ($p<0.05$).

have demonstrated that Roundup Ready corn event NK603 is equivalent in nutritive value to non-transgenic corn.

Natural Variability in Composition

The levels of individual biochemical components for agronomic crops are known to vary with genetic background and a variety of environmental factors such as climate, soil type, disease and insect infestation. One of the most

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dramatic examples of this phenomenon has been demonstrated by a study of the variation of isoflavone levels in a group of four different varieties of conventional soybeans raised at the same location in Urbana, Illinois and a single variety raised in four different locations in Illinois (18). The levels of total isoflavones were shown to vary two fold depending on the genetic background of the variety and almost four fold depending on the location. This degree of variation indicates the importance of controlled field trials where the biotech crop and the non-transgenic control are raised under identical conditions. It also suggests that understanding the natural variation in the levels of nutrients for the population of conventional commercial varieties is an essential feature in interpreting any differences observed between a biotech crop and its non-transgenic control.

The results from the analyses of conventional commercial varieties have been used to calculate a tolerance interval for the population of values for an individual biochemical component as illustrated for corn event NK603 in Table VII. A tolerance interval is an interval with a specified degree of confidence that contains a specified proportion of the entire population for the parameter measured. A comparison of the range of values for a biochemical component in the biotech crop with the tolerance interval for that component in the conventional varieties provides an indication if the biotech crop falls within the population of conventional commercial varieties for the same crop.

Conclusions

Extensive composition analyses have demonstrated that Roundup Ready soybeans and Roundup Ready corn event NK603 are substantially equivalent and therefore as safe as conventional corn currently in the marketplace. The samples for these analyses were obtained from controlled field trials, covering diverse geographies and multiple years. This extensive biochemical sampling, although directed towards nutritional parameters, also assesses unexpected effects due to the expression or the insertion of the introduced genes. In addition, the compositional profile of Roundup Ready corn event NK603 was compared to conventional corn varieties by calculating a 99% tolerance interval to describe the natural compositional variability present in the population of traditional corn varieties. All compositional values for corn event NK603 were shown to fall within the 99% tolerance interval for conventional corn which establishes that NK603 was within the same population as conventional corn. Compositional data in combination with agronomic and feed

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performance evaluations and safety evaluations of the introduced traits have established that Roundup Ready soybeans and Roundup Ready corn event NK603 are as safe and nutritious as conventional crops.

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Chapter 12

Biotechnology Crops as Feeds for Livestock

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Corn grain, whole plant green chop corn, corn silage, corn residue, soybeans, soybean meal, canola meal, cottonseed, sugar beets, fodder beets, and beet pulp from the currently marketed biotech crops are similar in nutrient composition, digestibility, and feeding value to their near isogenic parental lines when fed to chickens, pigs, sheep, dairy cows, and beef cattle. Therefore, growth, milk production, efficiency of feed utilization, and health of livestock are not different when they are fed biotech or conventional crops. The nutrient content of milk, meat, and eggs are not different when livestock are fed biotech or conventional crops. Foods produced by livestock fed biotech crops are safe for human consumption.

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Plants that supply feeds for livestock have improved over the years because new plant varieties were developed using conventional techniques of plant breeding. Biotech crops that supply feed for livestock produced through genetic enhancement are emerging from research and development to the marketplace because scientists have developed techniques to transfer specific genes from one organism to another, allowing the expression of desirable traits in the recipient organism. Crops that have been altered using modern technology are referred to as genetically modified, genetically enhanced, or biotech crops. To date, biotech crops that have reached the marketplace are based on producing insecticidal compounds or developing herbicide tolerance.

Corn grain, whole plant green chop corn, corn silage, corn residue, soybeans, soybean meal, canola meal, cottonseed, sugar beets, fodder beets, and beet pulp from the current biotech crops have been fed to livestock and compared with feeds produced from isolines of non-genetically enhanced plants. Chickens, pigs, sheep, beef cattle, and dairy cows have been used in these experiments. The purposes of these experiments were to compare biotech and conventional corn, soybeans, canola, cottonseed, and sugar beets for nutritional equivalence and digestibility, and to determine production and health of livestock fed these feeds. The objective of this paper is to review the results from experiments in which biotech crops were fed to livestock. Two previous reviews have been published on this topic (1, 2).

Insect-Protected (Bt) Corn

Plants that are genetically enhanced to contain a gene from *Bacillus thuringiensis* (Bt), a soil bacterium, produces a protein that affects only a narrow range of pests. This protein has insecticidal effects against the European corn borer, which is a common pest in corn fields. Corn borers reduce the quality and yield of corn and damage the plant tissue resulting in increased opportunity for fungal growth. Genetic enhancement to produce Bt corn that is resistant to this pest may improve the yield and the safety of corn for animal and human consumption by reducing fungal growth. This biotech corn has been compared with conventional corn in research trials with livestock to determine their nutritive value.

Chickens fed Bt Corn

Aulrich et al. (3) conducted a five-day feeding trial in which either Bt or non-Bt corn of an isoline (Cesar) was fed to laying hens. There were six hens per treatment and corn supplied 50% of the diet. Nutrient composition, including crude protein (CP), fat, Lys, Met, Cys, Ca, P, Mg, and fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$), was substantially equivalent for the Bt and non-Bt corns and diets. Digestibilities of organic matter (OM) and CP, and metabolizable energy (ME) content of the corns and diets were not different. Therefore, body weight of the hens did not change.

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German scientists (4) also conducted a 35-day feeding trial in which either Bt or non-Bt corn of an isoline (Cesar) was fed to broilers. There were 12 male chicks per treatment and 50% of the diet was corn. There were no significant differences between treatments for feed intake, feed conversion, CP digestibility, or body weight of the chicks at the beginning or end of the trial.

Non-Bt and Bt corns were compared at North Carolina State University in a trial with broiler chicks from 1 to 38 days of age (5). The experimental design was a 2 x 2 x 2 factorial consisting of mash versus pellets, males versus females, and non-Bt versus Bt corn. The Bt corn was from Event 176-Hybrid 5506 BTX and the isoline was G4665. There were 32 pens with 40 birds per pen. There were only minor differences in the moisture, fat, protein, fiber, ash, and amino acid contents of the non-Bt and Bt corns. Final body weight and the percentage of birds alive at the end of the trial were not significantly different for the non-Bt and Bt treatments. Birds that were fed diets that contained Bt corn had the best feed conversion ratio but this improvement can not necessarily be attributed to the source of corn because there were minor differences in the nutrient content of the diets. Most carcass components were not affected by the source of corn but the birds fed the Bt corn had a significant increase in breast skin and Pectoralis minor yields. Although the improved feed conversion and increased breast skin and Pectoralis minor yields cannot necessarily be attributed to the Bt corn per se, it does indicate that the Bt corn did not have detrimental effects on feed conversion and chick growth.

Ash (1.2 vs. 1.3%), crude fat (3.7 vs. 3.8%), CP (8.0 vs. 8.9%), and moisture (12.5% vs. 14.5%) contents of Bt and non-Bt corn were reported by Mireles et al. (6). They used nine replicate cockerels per treatment and indicated that, when corrected to an equal moisture content of 14.5%, true ME was 3,516 and 3,505 kcal/kg and the average amino acid digestibility was 89.5 and 90.7% for Bt and non-Bt corns, respectively. In a second trial, isocaloric and isonitrogenous diets that contained Bt or non-Bt corn were fed to six replicate cages of eight chicks per treatment for 21 days. Body weight gain (1,123 vs. 1,056 g/chick) and feed per gain (1.63 vs. 1.62) were not different. These nutritionists concluded that the nutritional value of Bt and non-Bt corn was similar when fed to broilers.

Diets of only corn supplemented with minerals and vitamins were fed to 210 broiler chicks with 6 birds per pen to determine the apparent ME content of Bt (MON810) and non-Bt corn (7). There was no difference in apparent ME content of the corn hybrids. This trial was followed by a 14-day growth assay conducted using 3-day old male broiler chicks in 50 pens with six birds per pen in a complete randomized design to evaluate Bt (MON 810), non-Bt, and three commercial corn hybrids (7). All diets contained the same percentage of corn. Average daily gain (ADG) of the chicks fed the five diets was not different; however, birds fed the Bt corn had a higher average daily feed intake and birds fed one of the commercial hybrids tended to have a lower gain to feed ratio. Bt corn was considered to be nutritionally equivalent to its near isogenic parent hybrid.

Italian scientists (8) compared Bt (MON810) and isogenic non-Bt corn grown in three locations. Nutritional composition was not different. The corn was fed to 432 Ross male broilers with 18 birds/pen for each treatment. Diets contained about

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50% corn and were formulated to meet requirements of the birds. Feed intake, ADG, and feed:gain of birds did not differ for birds fed these two sources of corn. Final live weight was 2.7% greater for birds fed the Bt corn. This increase in final live weight could be because of a 72% lower concentration of fumonisin B_1 in the Bt corn.

YieldGard® corn, YieldGard® plus Roundup Ready® corn, their parental lines, and four commercial hybrids were compared using broiler (Cobb x Cobb) chicks (5 male and 5 female pens/treatment with 10 birds/pen) in a randomized complete block design (9). Corn supplied about 50% of the diet from days 1 to 20 and about 60% of the diet from days 20 to 42. Feed and water were provided ad libitum. Data indicate there were no biologically relevant differences in chick performance, carcass yield, and meat composition among broilers fed the different sources of corn. These scientists concluded that these biotech corns were nutritionally equivalent to their parental lines and to the four commercial hybrids when fed to broilers.

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Pigs Fed Bt Corn

Digestibility trials were conducted with 20 crossbred barrows that averaged 26.4 kg of body weight to compare the nutritive value of Bt corn, a near isogenic parental hybrid, and three commercial hybrids (10). Digestible energy coefficients were not different between Bt corn and its near isogenic parent line; however, there were significant differences among other treatments.

Nutrient digestibility of two corn varieties, Cesar (Zea Mays L Line CG 00256-176) and a genetically enhanced Bt corn of the same line, were determined using five German Landrace barrows per treatment (11). The pigs weighed 40 kg and were fed diets (50% corn) that supplied 2.3 times their ME requirements during the experimental period. Digestibility of OM, CP, and nitrogen-free-extract (NFE), and ME content of the two sources of corn were not significantly different.

Bt corn (MON810) and its near isogenic parental line grown on four different farms were fed to 128 pigs (8.8 kg) divided into four treatments of 32 pigs each to compare their feeding value (12). Nutrient content of the Bt and control corn was not different. Pigs were fed diets that contained 33% corn for 35 days. Feed intake and feed consumed per unit of gain were not different. Average daily gain and final body weight were 5.6 and 2.8% higher, respectively, for pigs fed the Bt corn compared with the control corn. The increased body weight gain may be attributed to the Bt corn having a 69% lower concentration of fumonisin B_1 and a 14.4% lower concentration of deoxynevalenol than the control corn.

Bt corn (MON810), a near isogenic parental control, and a mixture of conventional non-Bt corn also were compared in a trial with 180 pigs (13, 14). The trial began when pigs weighed 30 kg and ended at slaughter when pigs weighed 121 kg. The experimental design was a 2 x 3 factorial of barrows versus gilts and three sources of corn. Source of corn had no effect on body weight gain, feed intake, or efficiency of feed utilization. Pigs fed Bt and the near isogenic control corns were

not different for hot carcass weight, dressing percentage, and predicted percent lean but fat depth at the 10th rib and loin marbling were greater for the near isogenic control. These scientists concluded that growth and carcass characteristics were similar for pigs fed Bt and the near isogenic control corns.

Composition and In Vitro Digestibility of Bt Corn Silage

Corn plants were collected from nine locations in Iowa, Illinois, Indiana, South Dakota, and Wisconsin to evaluate nutritive characteristics of fresh and ensiled whole plant material from several commercially available MON810 Bt corn hybrids and their respective non-Bt near-isogenic control hybrids (15, 16). Corn plants were harvested at 1/4 and 1/3 milk line and at the blacklayer stage of development. Whole plant material was chopped and ensiled using PVC mini silos. Nutrient composition and in vitro digestibility were determined on freshly chopped material and silage after 60 days of fermentation. When harvested at early blacklayer the fresh whole plant material from Bt hybrids had a higher moisture content, stayed green longer, and had a lower ammonia bound N content than the non-Bt hybrids. These scientists concluded that silage made from the Bt hybrids and their non-Bt near-isogenic hybrids were similar for nutrient composition and important feeding-related characteristics. In vitro digestibility of DM and cell walls from Bt and non-Bt corn silage harvested at 1/4 to 1/3 milk line and at early blacklayer stages of development were not significantly different. These findings suggest similar feeding values for silages made from Bt and non-Bt hybrids during all phases of typical corn silage maturity.

Lactating Dairy Cows fed Bt Corn Grain and Corn Silage

At Iowa State University, twelve lactating Holstein cows were used to investigate the feeding value of whole plant green chop from Bt and non-Bt corn hybrids (17; Faust, personal communication, June, 2000). Fresh, whole, green, chopped corn plants from two Bt corn hybrids (Event 176 and Bt 11) and from a control isogenic non-Bt hybrid were fed in diets of the cows for 14 days. Green chopped corn plants were fed to maximize the intake of the Bt protein. There were no significant differences among treatments for feed intake, milk production, or fat, protein, lactose, total solids, and urea in milk.

Sixteen lactating Holstein cows in a replicated 4 x 4 Latin square design with 21-day periods were used to evaluate the effects of early (N4242) and late (N7333) maturing corn with or without the Bt gene from Event Bt 11 at the University of Nebraska (18). Therefore, the four treatments were non-Bt early maturing corn, Bt early maturing corn, non-Bt late maturing corn, and Bt late maturing corn. The diets contained 40% corn silage, 28% corn grain from the same corn as the silage

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to maximize the hybrid effect, 10% alfalfa silage, and 22% of a protein, mineral, and vitamin mixture. There was no effect of the Bt trait in either the early or late maturing corn on dry matter intake (DMI), milk production, milk composition, milk component yields, fat-corrected milk (FCM) production, efficiency of FCM production, ruminal pH, concentration of volatile fatty acids (VFA) in rumen fluid, or in situ neutral detergent fiber (NDF) digestion kinetics.

Bt (Event 176) and non-Bt (isogenic Rh208) corn was grown in two locations in France and harvested as silage (19). Twenty-four dairy cows were fed either the Bt or non-Bt corn silage for 13 weeks in diets that contained 70% corn silage, 28% concentrate, and 1.9% wheat straw. Milk and 4% FCM production, CP, and fat composition of milk, and body weight change of cows were not affected by the source of corn silage. Dry matter intake was greater for cows fed the Bt corn silage. These scientists concluded that the feeding value of the Bt and non-Bt corn silages were equal.

In a second experiment, the French scientists (19) fed five midlactation Holstein cows either Bt or non-Bt corn silage for six consecutive weeks. Diets were fed as total mixed rations and contained 65.3% corn silage and 34.7% concentrate. During the third and sixth weeks of the experiment, milk samples were taken for detailed characterization of protein and fat fractions and for assessing coagulation properties related to cheese making. Nutrient composition of the two silages was similar. Milk yield; concentrations of protein, fat, and fatty acids in milk; and body weight change of cows were not different. The casein to protein ratio; the concentrations of casein, α-lactalbumin, β-lactoglobulin, NPN, and urea; rennet clotting time, rate of firming, curd firmness, and fresh curd yield also were not different. They concluded there was no difference in cheese-making ability of the milk from cows fed Bt and non-Bt corn silage.

Sheep Fed Bt Corn Silage

French scientists (19) compared Bt (Event 176) and non-Bt (isogenic Rh208) corn silages in a 15-day digestibility trial with seven Texel wethers. Net energy values for the corn silages fed at maintenance to the wethers and digestibilities of OM, crude fiber (CF), and NDF were not different for Bt and non-Bt corn silages.

German scientists (20) determined digestibility of Bt and non-Bt isogenic (Cesar) corn silage supplemented with protein using sheep. Four wethers were fed either Bt or non-Bt corn silage. Digestibilities of both silages were high and there were no significant differences between the silages for digestibility of OM, fat, CF, or NFE.

Feedlot Cattle Fed Bt Corn Silage and Corn Grain

Daenicke et al. (20) compared Bt and non-Bt isogenic (Cesar) corn silages as feeds for German Holstein bulls. Twenty bulls per treatment were assigned to a diet

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of either Bt or non-Bt corn silage plus a constant intake of concentrate. Bulls were about 165 days of age, initially weighed about 188 kg, and were fed corn silage until they weighed about 550 kg. There was no difference in the nutrient composition of the corn silages. Bulls fed Bt and non-Bt corn silages consumed the same amount of concentrate and similar amounts of as fed corn silage. Because the Bt silage was slightly lower in DM, bulls fed this silage consumed less DM and energy than bulls fed non-Bt silage. However, ADG, hot carcass weight, dressing percentage, and abdominal fat were not different for bulls fed the Bt and non-Bt corn silage.

To compare Bt (Event Bt 11) and non-Bt isogenic corn silages and early (N4242) and late (N7333) maturing corn silages, nutritionists at the University of Nebraska (18) assigned 128 steers that weighed 282 kg to a 2 x 2 factorial arrangement of treatments. Diets on a DM basis were 90% corn silage and 10% protein supplement (75% soybean meal and 25% urea on a N basis). The trial was 101 days in length. Dry matter intake was greater for steers fed Bt than non-Bt corn silage. There was a significant interaction between the Bt trait and the hybrid genotype for ADG and efficiency of feed utilization by steers. Average daily gain was greater for steers fed the Bt early maturing corn silage compared with the non-Bt early maturing corn silage but was similar for steers fed Bt and non-Bt late maturing corn silage. Efficiency of feed utilization was better for steers fed non-Bt than Bt late maturing corn silage but was similar for Bt and non-Bt early maturing corn silage. Incorporation of the Bt gene into the two different corn hybrids appeared to have different effects on performance of the steers. This effect was suggested to be related to the nutrient composition of the two corn hybrids from which the Bt corn was developed. Steers fed the early maturing corn silage gained faster and were more efficient in feed utilization than steers fed late maturing silage; however, the presence of the Bt gene in the corn hybrids did not consistently affect performance of the steers. Therefore, the genetics of the parent corn hybrid appeared to have a greater effect on animal performance than did the incorporation of the Bt gene into the corn.

Fifty-six steers (300 kg) were randomly allotted into eight pens of seven steers and fed whole plant corn silage during the growing phase and a high grain diet during the finishing phase to compare the feeding value of Bt corn (YieldGard®) and its near isogenic parental hybrid (Pioneer 3489 in year 1 and Pioneer 34E79 in year 2) during two cropping seasons (21). Steers were fed silage for 89 and 85 days and the finishing diet for 101 and 84 days, respectively, during years 1 and 2. The finishing diet on a DM basis contained 75% dry rolled corn, 15% corn silage, and 10% supplement. During year 1, source of silage did not affect DMI or ADG but efficiency of feed utilization was best for steers fed the control silage; however, during year 2 there was no difference in steer performance. There were no differences in steer performances during the finishing phase or in carcass characteristics at slaughter. These nutritionists concluded there were no major

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differences in the feeding value of Bt corn and its near isogenic parental hybrid when fed to beef steers.

Thirty-six crossbred steers were assigned to six pens and fed a 75% corn grain diet that contained 13% CP for 49 days during the terminal phase of the finishing period to compare the nutritional value of Bt corn (Event CBH351, Aventis) with conventional corn (22). The Bt and conventional corn contained 7.9% CP. Differences in DMI, ADG, gain to feed, and carcass yield and quality grades for steers fed Bt and conventional corn were not significant. Therefore, the Bt and conventional corn grains were not different in nutritional value for finishing beef cattle.

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Beef Cattle Grazing Bt Corn Residue

Experiments have been conducted to investigate the effects on beef cattle of grazing corn residue that contained the Bt trait. Russell et al. (23, 24) planted one non-Bt corn hybrid (Pioneer 3489) and three Bt-corn hybrids (Pioneer 34R07, YieldGard event; Novartis NX6236, YieldGard event; and Novartis N64-Z4, Knockout event) in duplicate 2.9 ha fields. After the harvest of grain, three mature cows in mid-gestation were assigned to duplicate fields for each treatment (six cows/treatment) to strip-graze for 126 days. Six cows also were assigned to duplicate drylots. All cows were fed alfalfa-grass hay to maintain a body condition score of five on a nine-point scale. There were no differences in yields of grain or dropped grain. Dry matter and OM contents and yields of in vitro digestible DM were not different for these sources of corn residue. Source of corn residue did not affect the mean rates of change in composition of corn residue during the 126 days of grazing. There were no significant differences in body weight or body condition score of cows among treatments. To maintain similar body condition scores, cows grazing corn residues required a smaller quantity of hay than did cows maintained in drylot. The amount of hay required to maintain body condition of the cows was not different for cows fed the non-Bt and the Bt corn residues.

Sixty-seven steers that weighed an average of 284 kg were used in a two part 70-day trial to evaluate their performance when fields of Bt N7333 and isogenic non-Bt N7333 corn residues were grazed (18). Fifty-one steers were assigned to the fields to achieve equal stocking rate per hectare for the Bt (27 steers) and non-Bt (24 steers) treatments. Sixteen additional steers were used to evaluate grazing preference for the Bt and non-Bt corn residue. Steers were fed 0.45 kg of protein supplement/head per day to ensure that protein did not limit animal performance. Average daily gain and grazing preference were not different for steers grazing Bt and non-Bt corn residues.

Hendrix et al. (25) used 78 nonlactating pregnant beef cows during two years for an average of 38 days per year to determine performance of cows grazing Bt or isogenic non-Bt corn residues. Twenty additional cows were given access to both

Bt and isogenic non-Bt corn residues in year one to determine grazing preference. There were no differences in average body weight change or choice of grazing preference by cows grazing Bt or non-Bt corn residue. There were no major differences in feeding value of these corn residues.

Chickens and Beef Steers Fed Root Worm Protected Corn

Ross x Ross 508 broiler chickens were fed diets that contained root worm protected corn (event MON863), its non-transgenic control, and commercial corn in a 42-day trial to compare performance, carcass yield, and meat composition (26). Diets were fed ad libitum and contained ~55% w/w corn for the first 20 days and ~60% w/w corn for the remainder of the trial. A randomized complete block design was used with 5 male and 5 female pens/treatment (10 birds/pen). Biologically relevant differences were not observed in broiler performance (body weight, feed intake, feed conversion), carcass yield, and meat composition, which indicated nutritional equivalence for these sources of corn.

Steers (365 kg) were fed Bt corn (Bt event MON863), a non-transgenic parental hybrid (RX670), or one of two non-transgenic commercial hybrids (RX740 or DK647) in a 112-day trial to evaluate the effects of root worm protected corn on performance and carcass characteristics (27). A completely random design was used with 10 steers/pen and five pens/treatment. Diets (DM basis) consisted of 77.5% corn, 10% steep liquor, 7.5% ground alfalfa, and 5% supplement. Steers fed DK647 consumed more DM than steers fed the other three sources of corn. Steers fed the Bt corn used feed more efficiently than steers fed the other sources of corn. Final weight, ADG, 12th rib fat thickness, and marbling score of the steers were not affected by treatment. Feeding value of these corns was suggested to be similar for feedlot steers.

Herbicide-Tolerant Crops

Herbicide-tolerant plants that are currently being marketed are produced by the stable insertion of a gene that expresses a glyphosate-tolerant, modified plant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein in the receptor plant (28) or by the insertion of the phosphinothricin-acetyl-transferase-gene (Pat-gene) from the bacteria *Streptomyces viridochromogenes* rendering the plants tolerant to the herbicides glyphosate and glufosinate, respectively. Glyphosate-tolerant crop varieties have been developed and commercialized for corn, soybean, canola, cotton (29), and sugar beets. Glufosinate-tolerant crop varieties have been developed for corn and sugar beets. Research trials have been conducted to investigate the

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nutrient composition and the feeding value of the herbicide-tolerant crops for food producing animals.

Composition of Glyphosate-Tolerant Corn

Grain and whole plant green chop from glyphosate-tolerant corn (GA21), a control parental line (DK626), and five commercial hybrids were analyzed for nutrient composition (29). Results of these analyses indicated that, except for a few small differences that probably are not biologically significant, moisture, CP, fat, ash, carbohydrate, ADF, NDF, amino acid, fatty acid, Ca, and P contents of the corn grain and whole plant green chop were not significantly different. Therefore, the glyphosate-tolerant corn grain and green chop were substantially equivalent in composition to the control and commercial corn hybrids.

Chickens Fed Glyphosate-Tolerant Corn

A control parental line (DK580), a glyphosate-tolerant line (GA21/DK580) and five commercial hybrids of corn were evaluated using 560 growing broiler chickens (80/treatment) in a 39-day growth trial (29). Growth, feed efficiency, feed efficiency adjusted for feed consumption of dead chickens, and fat pad weights were not different for chickens fed the control or glyphosate-tolerant corn. Likewise, these same measurements were not different for chickens fed the glyphosate-tolerant corn and the population mean for chickens fed the five commercial corn varieties.

Two experiments were conducted by Gaines et al. (7) to compare a near isogenic parental line, a glyphosate-tolerant line, and three commercial lines of corn. Growth was measured in a 14-day trial using 50 pens of 3-day old male broiler chicks with five chicks per pen in a complete randomized design. Apparent ME digestibility coefficients were determined using 35 pens of chicks with five chicks per pen. Differences among treatments were not significant for ADG, feed intake, gain to feed, and apparent metabolizable digestibility coefficients. The glyphosate-tolerant and the near isogenic parental corns were considered to be nutritionally equivalent.

Glyphosate-tolerant corn (Event NK603), its parental line, and five commercial lines of corn were compared using broiler (Ross x Ross) chicks (5 male and 5 female pens/treatment with 10 birds/pen) in a randomized complete block design (30). Corn supplied about 55% of the diet from days 1 to 20 and about 60% of the diet from days 20 to 42. Feed and water were provided ad libitum. Source of corn did not affect live weight; feed intake; feed efficiency; live weight per chill weight; breast meat, thighs, drums, and wings as percent of chill weight; or percentages of moisture, protein, and fat in breast meat or protein and fat in thigh meat. Small

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differences were observed among treatments for fat pad weights and moisture content of thigh meat. Conclusions were that the glyphosate-tolerant corn containing the NK603 event was nutritionally equivalent to the parental control and commercial lines when fed to broilers.

Pigs Fed Herbicide-Tolerant Corn

Digestible energy coefficients for diets that contained glyphosate-tolerant corn, its near isogenic parental hybrid, and three commercial corn hybrids were determined using 20 crossbred barrows that averaged 25.5 kg in body weight (10). There were significant differences among some treatments for digestible energy coefficients; however, the digestible energy coefficient for glyphosate-tolerant corn was not different from its near isogenic parental hybrid.

One hundred sixty pigs (80 barrows and 80 gilts) were fed four corn hybrids to determine their effects on growth and carcass characteristics during a period of growth from 72 to 117 kg of body weight (31). The four corn hybrids were glyphosate-tolerant corn (DK626RR, event GA21), its parental line DK626, Asgrow RX826, and corn purchased from a feed mill. Corn-soybean meal based diets and water were provided ad libitum. Each diet contained the same percentage of corn and all diets were standardized for amino acid content with crystalline amino acids. Average daily body weight gain, average daily feed intake, efficiency of feed utilization, and composition of muscle did not differ among treatments. Results indicate that these four sources of corn had a similar feeding value for pigs.

One hundred sixty pigs (80 barrows and 80 gilts) were raised from 29.9 to 119.4 kg of body weight in single sex groups of 5 pigs each (32). Pigs were fed diets that contained a glyphosate-tolerant corn hybrid (event NK603), a parental control line, and two commercial lines of non-genetically modified corn. All diets were formulated with a fixed concentration of corn inclusion which was 65, 74, and 77% for the growing- (30 to 50 kg of body weight), early- (50 to 80 kg), and late- (80 to 120 kg) finishing phases, respectively. Pigs fed the four corn lines had similar average daily feed intakes, ADG, and gain:feed ratios. Carcass measurements (dressing percentage, carcass length, backfat thickness, and longissimus muscle area) and subjective scores for longissimus muscle color, firmness, and marbling scores taken at the 10th rib were similar for pigs fed all sources of corn.

Diets that contained glyphosate-tolerant corn (event NK603), a non-transgenic control corn (RX670), or two commercial sources of non-transgenic corn (RX740 or DK647) were fed to 72 barrows and 72 gilts from an initial body weight of 22.6 kg to a final body weight of 116 kg (33). The experimental design was a randomized complete block design using a 2 x 4 factorial arrangement. Average daily gain, average daily feed intake, and gain:feed ratio were not affected by diet. Real-time ultrasound measurements, total body electrical conductivity

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measurements, and proximate analysis composition of longissimus muscle were similar for pigs fed the four diets.

German scientists (34) determined the nutrient content of conventional corn and two genetically enhanced glufosinate-tolerant hybrids. They reported that the CP, ether extract, carbohydrate, amino acid, fatty acid, and cell wall constituents were not different among these three sources of corn. Diets that contained 30% corn were fed to German Landrace barrows (5/treatment) in the growing period between 38 and 52 kg of live weight and feces were collected for 10 days proceeded by a 14-day adaptation period. Diets supplied 2.3 times the energy maintenance requirement. Data indicate that nutrient digestibility and feeding value of these corns were similar for pigs.

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Lactating Dairy Cows Fed Glyphosate-Tolerant Corn

Sixteen multiparous Holstein cows from 71 to 107 days in milk were assigned to two groups and used in a switchback design with three periods of 28 days each to evaluate the feeding value of glyphosate-tolerant corn (GA21) or a control isogenic line (DK626) of corn (Donkin, personal communication, June, 2000). Cows were fed for ad libitum intake diets that contained 62% corn silage, 17% corn grain, and 21% protein, mineral, and vitamin supplement. There were no differences in DMI, milk production, 4% FCM production, somatic cell count, milk urea N, or percentages and yields of protein, fat, lactose, and solids-not-fat in milk when cows were fed glyphosate-tolerant corn or the isogenic line of corn.

Sixteen multiparous Holstein cows that averaged 74 days postpartum were used in a replicated 4 x 4 Latin square to compare the effects on animal performance of feeding whole plant silage and grain from a glyphosate-tolerant corn hybrid (even NK603), the non-transgenic control line, and two commercial non-transgenic hybrids (DK647 and RX740) (35). All diets contained 30% corn silage and 27.34% corn grain (DM basis). Feeding diets that contained event NK603 (24.6 kg/day) and DK647 (24.5 kg/day) hybrids tended ($P < 0.06$) to decrease DMI compared with the control line (25.5 kg/day) and the RX740 (26.1 kg/day). Production of milk, 3.5% FCM, fat, crude protein, true protein, and total solids and the percentages of milk fat, crude protein, true protein, and total solids, as well as milk urea N and somatic cell count were not affected by treatments. These data indicate that the stable insertion of the gene that confers tolerance to glyphosate in corn (event NK603) used in this experiment does not affect lactation performance of dairy cows.

To compare a glyphosate-tolerant corn hybrid (event NK603) with its non-transgenic parental control and two reference hybrids, 16 multiparous Holstein cows (95 days postpartum) were fed one of four diets in a replicated 4 x 4 Latin square design (36). The diets contained 40% corn silage and 23% corn grain (DM basis) from either a glyphosate-tolerant corn, a non-transgenic parental control, or

two commercial reference hybrids. Chemical composition was similar among the four corn hybrids for grain and silage, except for the DM content of the glyphosate-tolerant corn silage. The glyphosate-tolerant corn silage was harvested drier (42.5% DM) and fermentation of this silage was less extensive than for the wetter control corn silages (35.5% DM). Dry matter intake and milk production were decreased by 14 and 12%, respectively, for cows fed the glyphosate-tolerant diet compared with the other three diets. However, efficiency of 4% FCM production, milk urea nitrogen; somatic cell count; and percentages of fat, true protein, lactose, and solids-not-fat in milk were not affected by diet.

Feedlot Cattle Fed Glyphosate-Tolerant Corn

To compare glyphosate-tolerant corn (DK626RR; GA21) and its near isogenic parental hybrid (DK 626), 56 steers (304 kg) were randomly allotted into eight pens of seven steers (37). Four pens of steers were fed a diet of 90% whole plant corn silage from each corn plus 10% supplement on a DM basis for 85 days during the growing period and four pens of steers were fed a diet of 75% dry rolled corn and 15% whole plant corn silage from each corn plus 10% supplement on a DM basis for 84 days during the finishing period. Differences for ADG, DMI, and efficiency of feed utilization were not significant during the growing period, finishing period, or the two combined periods. At slaughter, there were no differences in carcass characteristics. Results indicate that the feeding value of silage and grain from the glyphosate-tolerant corn and its near isogenic hybrid are equal when fed to growing and finishing beef cattle.

The effects of glyphosate-tolerant corn (event NK603) on performance and carcass characteristics of finishing steers (321 kg) were determined in a 144-day experiment (38). A completely randomized design was used with 10 steers/pen and five pens/treatment. Steers were fed diets that contained either glyphosate-tolerant corn, a non-transgenic control corn hybrid (RX670), or one of two non-transgenic commercial hybrids (RX740 or DK647). Diets (DM basis) consisted of 79.5% corn, 10% steep liquor, 7.5% ground alfalfa, and 3% supplement. Initial weight and final weight of steers, DMI, ADG, efficiency of feed utilization, carcass characteristics, and meat composition were not different among treatments.

Feedlot steers were used in two trials at the University of Illinois to evaluate the effect of glyphosate-tolerant corn on their performance and carcass characteristics (39). Angus-Continental steers were fed diets in Trial 1 that contained grain from glyphosate-tolerant corn (DK626, event GA21), non-transgenic control (DK626), and one of two commercial hybrids (RX826 or RX730). Steers were assigned by weight to one of 25 pens (7 steers/pen) in a randomized complete block design. Treatment effects were not different for ADG, DMI, efficiency of feed utilization, or carcass characteristics. Percent moisture of the longissimus thoracic muscle was greater for steers fed the non-transgenic

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control compared with other diets but protein, ash, and ether extract were not different among treatments. In Trial 2, 196 Continental-cross steers were assigned by weight to one of 28 pens (7 steers/pen). Steers were fed a glyphosate-tolerant corn hybrid (event NK603), a non-transgenic control, or one of two conventional hybrids (DK647 or RX740). Steers fed RX740 had the highest DMI (10.49 kg/day) which was greater than for steers fed DK647 (9.92 kg/day). Steers fed the non-transgenic control (10.02 kg/day) and glyphosate-tolerant corn (10.18 kg/day) were intermediate. Differences among treatments were not significant for ADG, efficiency of feed utilization, or carcass characteristics. It was concluded that the glyphosate-tolerant corn was similar to the other sources of corn in nutritive value for feedlot steers.

Composition of Herbicide-Tolerant Soybeans

Padgette et al. (40) investigated the composition of two glyphosate-tolerant soybeans (40-3-2 and 61-67-1) and a control parental soybean variety (A5403) from 13 fields during two years. Nutrients measured in the soybean seeds included nutrients by proximate analyses (CP, fat, fiber, ash, carbohydrates), amino acids, and fatty acids. Antinutrients measured in the soybean seed or toasted meal were trypsin-inhibitor, lectins, isoflavones, stachyose, raffinose, and phytate. Nutrients by proximate analyses were determined for defatted toasted meal, defatted nontoasted meal, protein isolate, and protein concentrate prepared from the three soybean varieties. Fatty acid composition of soybean oil from the three varieties also was measured. Significant differences were detected between the control and glyphosate-tolerant soybean seeds for some of the nutrients determined by proximate analyses. However, these differences were small and were considered biologically unimportant. There were no significant differences in anti-nutrient, amino acid, or fatty acid content. These data indicated that the composition of control and glyphosate-tolerant soybeans were substantially equivalent.

Chickens Fed Glyphosate-Tolerant Soybeans

Three hundred and sixty broiler chickens were used from birth to 42 days of age to evaluate the feeding value of soybean meal produced from two glyphosate-tolerant soybeans (40-3-2 and 61-67-1) and a control parental variety (A5403) (41). The experimental design was a 3 x 2 factorial arrangement with three soybean varieties and two sexes of chickens (equal numbers). At the end of the 42-day experiment there were no significant differences among sources of soybean meal for body weight, live weight gain, feed intake, gain:feed ratio, survival, breast muscle, or fat pad weight of chickens.

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Pigs Fed Glyphosate-Tolerant Soybeans

Dehulled soybean meal prepared from glyphosate-tolerant soybeans or from near isogenic conventional soybeans were compared in an experiment with growing-finishing pigs (42). Nutrient composition of the two sources of soybean meal were similar. Crossbred pigs were allotted to 10 pens (5 pens each of barrows and gilts) per treatment with 5 pigs/pen and fed corn-soybean meal based diets from 24 to 111 kg of body weight. The ADG, average daily feed intake, efficiency of feed utilization, scanned backfat and longissimus area, and calculated carcass lean were not different between treatments. Dressing percentage, backfat at the 10th rib, and longissimus area for barrows at slaughter also were not different between treatments. Samples of longissimus from barrows fed conventional soybean meal tended to have less fat than barrows fed enhanced soybean meal but water, protein, and ash were similar. Results indicate the glyphosate-tolerant soybean meal is essentially equivalent in nutrient composition and feeding value for growing-finishing pigs.

Lactating Dairy Cows Fed Glyphosate-Tolerant Soybeans

To evaluate glyphosate-tolerant soybeans, thirty-six multiparous Holstein cows ranging from 93 to 196 days in milk at the start of the experiment were fed total mixed rations that contained 10.2% of one of three whole raw soybeans on a DM basis (41). The soybeans were two lines of glyphosate-tolerant (40-3-2 and 61-67-1) and a control parental variety (A5403). The trial was 29 days in length with digestibility and N balance determined from day 21 to 28 and ammonia and VFA concentrations in rumen fluid determined on day 29. Differences among treatments were not significant for DM, net energy for lactation (NE_L), or N intakes; milk production; 3.5% FCM/NE_L; percentages of protein, fat, or lactose in milk; somatic cell count; DM digestibility; N absorbed or retained; N excreted in feces and urine; and concentration of ammonia N or molar percentages of VFA in ruminal fluid. Production of 3.5% FCM was greater for cows fed glyphosate-tolerant soybeans because both milk production and milk fat percentage were slightly but not significantly greater than for cows fed the control parental variety. These data indicate that the feeding value of these sources of soybeans are substantially equivalent.

Chickens and Lambs Fed Glyphosate-Tolerant Canola Meal

A 42-day trial with growing Ross x Ross 508 broilers was used to compare meal from glyphosate-tolerant canola (event RT73) with a non-transgenic control

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and six commercial lines (43). Starter and grower diets contained 25 and 20% canola, respectively, and were supplemented with soybean meal and corn to meet requirements. A randomized complete block design with 5 pens of males and 5 pens of females per treatment and 10 birds per pen was used. These scientists concluded there were no biologically relevant differences in performance or carcass quality between broilers fed glyphosate-tolerant canola, its control, or commercial lines.

Two experiments were conducted to evaluate the effects of including meal from glyphosate-tolerant canola in barley-based diets for lambs (44). Four diets that contained glyphosate-tolerant canola meal, the non-transgenic parental control meal, and one of two meals from commercial canola lines were fed to the lambs. In trial 1, 60 early-weaned Canadian Arcott lambs (30 ewes and 30 wethers; ~2 months of age) were fed the diets from an initial weight of 21.5 kg to at least 45 kg. The lambs were individually penned, blocked by initial weight, and gender for assignment to treatments. In trial 2, apparent digestibilities of the four diets were measured using eight mature wethers (67.8 kg) in a replicated Latin square. In these two trials, glyphosate-tolerant canola meal did not alter digestibility (DM, fiber), nitrogen balance, efficiency of feed utilization, growth, carcass characteristics, or meat quality of the lambs.

Insect-Protected and Herbicide-Tolerant Corn and Cotton

Performance, carcass yield, and meat composition were compared in a 42-day trial in which Ross x Ross 508 broiler chickens were fed diets that contained YieldGard® (event MON810) x glyphosate-tolerant (event NK603) corn, a non-transgenic control corn, or commercial corn (45). Diets contained ~55% w/w corn for the first 20 days and ~60% w/w corn thereafter. Chickens were assigned to eight treatments (5 male and 5 female pens/treatment and 10 chickens/pen) in a randomized complete block design. Broilers fed the diets that contained biotech corn, non-transgenic control corn, or commercial corn showed no biologically relevant differences in performance (body weight, feed intake, feed conversion), carcass yield, and meat composition. Therefore, this insect and herbicide tolerant corn was substantially equivalent to its non-transgenic control and other commercial corn hybrids.

In addition to the herbicide tolerance for glyphosate, the cotton plant has been genetically enhanced with the Bt gene to protect it from the bollworm which destroys bolls or unripe pods of cotton. Scientists (46) in Argentina compared the feeding value of genetically enhanced and control cottonseed for lactating Holstein cows. Twelve cows were used in a 4 x 4 Latin square design with four periods of

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four weeks each. The four treatments were cottonseeds that were not genetically enhanced, Bollgard® Cottonseed (Cry1Ac), Bollgard® II Cottonseed (Cry1Ac and Cry2Ab), and Roundup Ready® Cottonseed (glyphosate-tolerant, CP4 EPSPS). All cows were fed a diet of corn silage, alfalfa hay, ground corn, soybean meal, minerals, vitamins, and 2.27 kg of whole cottonseed. There were no significant differences among treatments for DMI, cottonseed intake, milk yield, milk composition or body condition score.

Conventional and biotech cottonseed also were compared in a second experiment in Argentina (47). Twelve lactating Holstein cows were used in a 4 x 4 Latin square design with four week periods. The four treatments were three nongenetically enhanced varieties consisting of two commercial sources (Guazuucho and Pora), a parental line (Chaco 520), and the biotech cottonseed (Chaco 520 BGRR) that contained both the Bollgard® (Cry1Ac) and Roundup Ready® genes. Cows were fed a diet of corn silage, alfalfa hay, ground corn, soybean meal, minerals, vitamins, and 2.27 kg of cottonseed. Dry matter intake, cottonseed intake, milk yield, milk composition, and body condition score were not different for cows fed these sources of cottonseed. Results of these experiments indicate that cottonseed that contained Bollgard®, Bollgard® II, and Roundup Ready® genes provide similar nutritive value as conventional cottonseed when fed to lactating dairy cows.

Herbicide-Tolerant Sugar Beets

Böhme et al. (34) grew control sugar beets and glufosinate-tolerant sugar beets that were either managed conventionally or sprayed with glufosinate. These sugar beets and silage from the foliage of these sugar beets were assayed for DM, OM, CP, ether extract, CF, and NFE and their composition was similar. The sugar beets also contained a similar sugar content.

Nutrient digestibility and energetic feeding value of the three sugar beets and sugar beet foliage silages were determined using pigs and sheep (34). Fifteen barrows (5/treatment) were fed diets that contained the sugar beets (30% of dietary DM) in the growing period between 38 and 52 kg live weight. The diets supplied 2.3 times the pigs maintenance requirement. Small but significant increases in OM digestibility were obtained when the Pat-lines of sugar beets were fed to the pigs but digestibilities of CP and NFE, and ME concentrations were not different among treatments. Twelve wethers (95 kg body weight; 4/treatment) were fed diets that contained either 50% sugar beets or 60% sugar beet foliage silage and nutrient digestibility and energetic feeding values were determined. Differences among the three sugar beets fed to the wethers were not significant for digestibilities of OM, CP, CF, and NFE, or for ME content. Differences among the three sugar beet foliage silages fed to the wethers were not significant for OM or CP; however, minor but significant differences were obtained between the silages with the Pat-

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gene and the control silage for digestibility of ether extract, CF, and NFE, and for ME content. It was concluded that these minor differences were biologically unimportant and that these biotech and control sugar beets were substantially equivalent.

Danish scientists (48) compared six varieties of sugar beets (5 control + 1 glyphosate-tolerant), five varieties of fodder beets (4 control + 1 glyphosate-tolerant), and six varieties of beet pulp (5 control + 1 glyphosate-tolerant) in three digestibility trials with seven sheep/treatment fed at maintenance. It was concluded that glyphosate-tolerant sugar and fodder beets, or pulp derived from glyphosate-tolerant sugar beets, have a nutrient digestibility that is within the range observed for conventional beets and pulps, indicating that feeding value is not affected by rendering the beets glyphosate-tolerant.

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Commercially Grown and Utilized Biotech Crops

Modern methods of biotechnology are being accepted and used by farmers who planted about 44.2 million hectares of biotech crops globally in 2000 (49). From 1996 to 2000 the global hectares of biotech crops increased from 1.7 million hectares to 44.2 million hectares or about 2500% (49). About 19.5 million hectares of biotech soybeans (63%), 7.5 million hectares of biotech corn (24%), and 4.0 million hectares of biotech cotton (64%) were to be planted in the United States in 2001 (50). Approximately 70% of the soybeans produced in the world (51) and 80% of the corn produced in the United States (52) are consumed by animals. Since these biotech crops were grown beginning in 1996, they have been fed to livestock. No detrimental effects have been reported when these feeds were fed to livestock, which supports findings from the research trials summarized above.

Benefits, Food Supply, and Safety Issues Associated with Biotech Crops

It has been estimated that the world's population will increase from the current six billion people to about 10 billion people by the year 2040. It also has been estimated that the supply of food required to adequately meet human nutritional needs over the next 40 years is quantitatively equal to the amount of food previously produced throughout the entire history of humankind (53). If we are to adequately feed this growing population, modern methods of biotechnology must be used to produce crops that supply feed for livestock and food for humans. We must be sure that these and future products produced using modern techniques of biotechnology are safe for both livestock and humans if they are to be eaten now and in the future. In this regard, Beever and Kemp (54) in an excellent review

concluded "Additionally there is a growing body of scientifically valid information available that indicates no significant risk associated with the consumption of DNA or the resulting proteins from GM crops that are registered in any of these countries (European Union, USA, Japan, Australia, Canada, Argentina, other countries). Based on the safety analyses required for each crop, consumption of milk, meat and eggs produced from animals fed GM crops should be considered to be as safe as traditional practices." The regulatory review process to assess safety of biotech crops for animals and the food chain, and the many benefits of biotech crops have been discussed previously (55, 56, 57, 58, 59, 60, 61, 62).

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