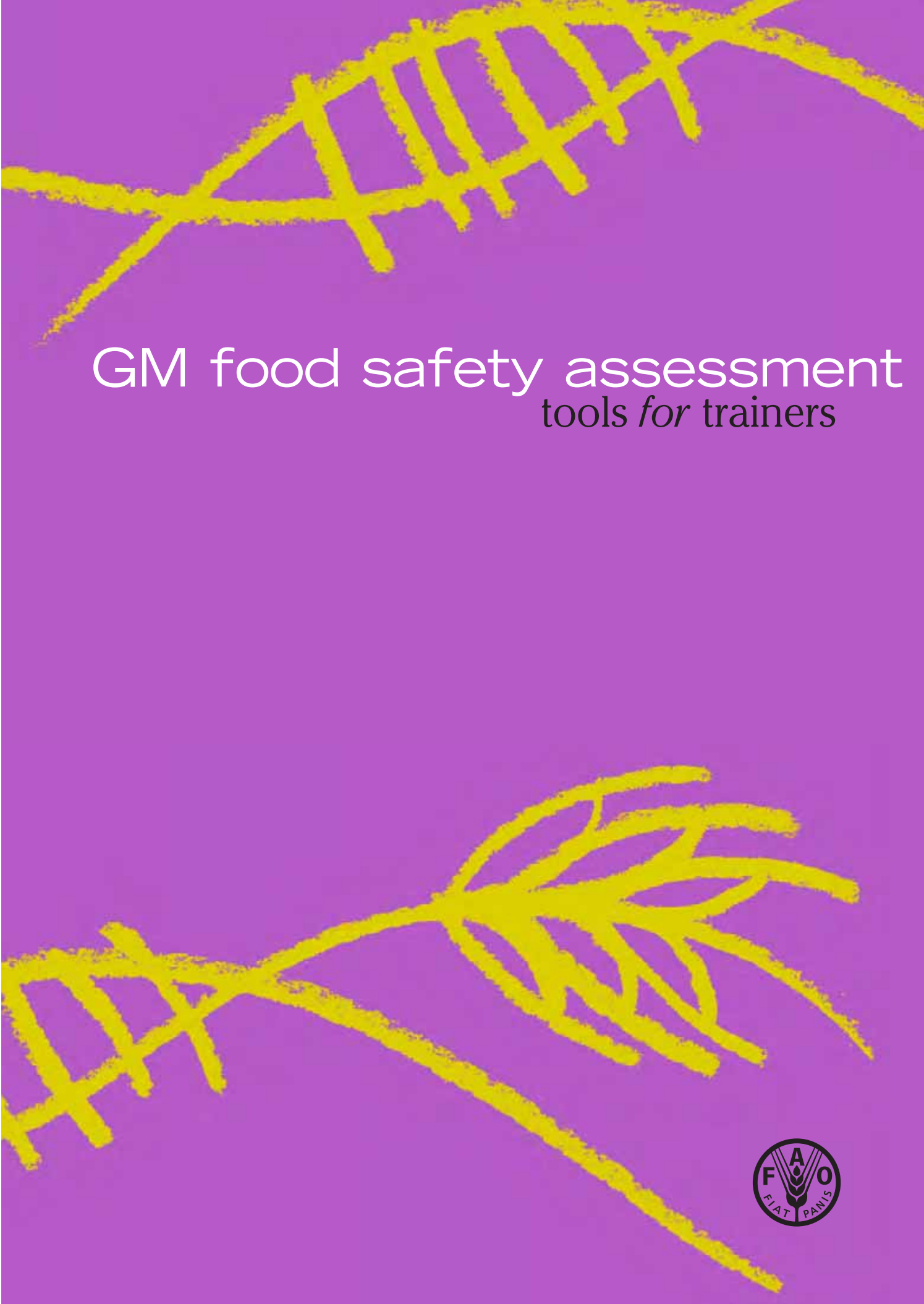
GM food safety assessment

tools *for* trainers



GM food safety assessment

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Food and Agriculture
Organization
of the United Nations
Rome, 2008

ISBN 978-92-5-105978-4

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Acknowledgenments

FAO would like to express its appreciation to the many people who provided advice and guidance during the preparation of this publication. This training tool was prepared for the Food Quality and Standards Service (AGNS) of the Food and Agriculture Organization of the United Nations (FAO). The original document was drafted by the FAO international consultant, Morven McLean, Ph.D., and further developed by Masami Takeuchi, Ph.D., AGNS/FAO, and Ezzeddine Boutrif, Director, Nutrition and Consumer Protection Division (AGN). Several people in AGNS and other units in FAO provided comments and suggestions and their inputs are gratefully recognized. The tool was proof-read and edited by Sarah Binns.

The Canadian Government, represented by Health Canada, was actively involved in contributing to the initial draft and implementing the training at the pilot-testing workshop. FAO would like to thank William Yan, Health Canada, Paul Brent, Food Standards Australia and New Zealand (FSANZ) and Kathleen Jones, United States Food and Drug Administration (US FDA), who were also involved in improving the initial draft before pilot-testing the tool. It is appreciated that a number of experts from different parts of the world in the field of safety assessment of foods derived from modern biotechnology were involved in the pilot testing, which was held in Ottawa, Canada in 2006. FAO is also grateful to the international experts who participated in the final peer review meeting held in Bangkok in 2007, namely Behzad Ghareyazie, Sathin Kunawasen, Kelebohile Lekoape, Kaare M. Nielsen, Marilia Nutti, Vinod Prabhu and Ruud Valyasevi, for their interest and commitment, and for their valuable contributions to greatly improve the tool. Last, but not least, FAO would like to thank the Government of Norway, which provided financial support for the development and publication of this training tool under the FAO Norway Partnership Programme ●

Foreword

The Food and Agriculture Organization of the United Nations (FAO) recognizes that biotechnology provides powerful tools for the sustainable development of agriculture, fisheries and forestry, as well as the food industry. When appropriately integrated with other technologies for the production of food, agricultural products and services, biotechnology can be of significant assistance in meeting the needs of an expanding and increasingly urbanized population in the next millennium.

There is a wide array of "biotechnologies" with different techniques and applications. The Convention on Biological Diversity (CBD) defines biotechnology as:

> *any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use.*

When interpreted in this broad sense, the definition of biotechnology covers many of the tools and techniques that are commonplace in agriculture and in food production. Interpreted in a more narrow sense, the definition covers specific technologies such as gene modification and transfer, DNA typing and cloning of plants and animals. The definition of modern biotechnology for the purpose of food biosafety analysis is, however, explicitly used for foods derived from genetic engineering and fusion of cells beyond taxonomic families, as adopted from the Cartagena protocol on biosafety by the Codex Alimentarius Commission (CAC). The definitions of biotechnology and modern biotechnology referred to in this document can be found in the Glossary of the Tool.

While there is little controversy about many aspects of biotechnology and its application, recombinant-DNA derived plants, also referred to as genetically modified organisms (GMOs), living modified organisms (LMOs, under the Cartagena Protocol of CBD), genetically engineered crops and transgenic crops, have become the target of a very intensive and, at times, emotionally charged debate. FAO recognizes that genetic engineering has the potential to help increase production and productivity in agriculture, forestry and fisheries. However, FAO is also aware of the concern about the potential risks posed by certain aspects of modern biotechnology. These risks fall into two basic categories: the effects on human and animal health and the environmental consequences. Care must be taken to reduce the risks of transferring toxins from one life form to another, of creating new toxins or of transferring allergenic compounds from one species to another, which could result in unexpected allergic reactions. Risks to the environment include the possibility of outcrossing, which could lead, for example, to the development of increased plant weediness or wild relatives with increased resistance to diseases or environmental stresses, thus upsetting the balance of the ecosystem. As in the case of growing any improved cultivar with improved traits, biodiversity may also be lost, for example as a result of the displacement of traditional cultivars by a small number of genetically modified cultivars.

FAO supports a science-based evaluation system that would determine the benefits and risks of each individual GMO. This calls for a case-by-case approach to address the concerns regarding the biosafety of each product or process prior to its release. The possible effects on

biodiversity, the environment and food safety need to be evaluated, and the extent to which the benefits of the product or process outweigh its risks must be assessed. The evaluation process should also take into consideration experience gained by national regulatory authorities in clearing such products. Careful monitoring of the post-release effects of these products and processes is also essential to ensure their continued safety to human beings, animals and the environment.

Current investment in biotechnological research tends to be concentrated in the private sector and oriented towards agriculture in higher-income countries where there is purchasing power for its products. In view of the potential contribution of biotechnologies to increasing food supply and overcoming food insecurity and vulnerability, FAO considers that efforts should be made to ensure that developing countries, in general, and resource-poor farmers, in particular, benefit more from biotechnological research, while continuing to have access to diverse sources of genetic material. FAO recommends that this need should be addressed through increased public funding and dialogue between the public and private sectors.

FAO continues to assist its member countries, particularly developing countries, to reap the benefits derived from the application of biotechnologies in agriculture, forestry and fisheries. It also assists developing countries to participate more effectively and equitably in the trade in international commodities and food. FAO provides technical information and assistance, as well as socio-economic and environmental analyses, on major global issues related to new technological developments. For instance, together with the World Health Organization (WHO), FAO provides the secretariat to the Codex Alimentarius Commission (CAC), which has established an *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnologies (TFFBT). Government-designated experts in the task force will develop standards, guidelines or recommendations, as appropriate, for foods derived from biotechnologies or traits introduced into foods by biotechnological methods. The CAC is also considering approaches that will allow the consumer to make informed choices.

FAO is constantly striving to determine the potential benefits and possible risks associated with the application of modern technologies to increasing plant and animal productivity and production. However, the responsibility for formulating policies towards these technologies rests with the member governments themselves. To be in a position to take full advantage of the technology, countries must have the necessary infrastructure, financial support and expertise. In the case of GMOs, countries will also need to put the necessary regulatory framework in place to minimize potential risks. To this end, FAO provides technical advice for the establishment of appropriate regulatory frameworks in the fields of biosafety, food safety and intellectual property rights.

We welcome comments and feedback on this training tool as part of our ongoing commitment to support member countries to strengthen their capacity to assess the safety of foods derived from modern biotechnology and to manage better all relevant issues in protecting public health, agricultural production and the environment, in the concept of "Biosafety[1] within the Biosecurity[2] framework" ●

Ezzeddine Boutrif
Director, Nutirition and Consumer Protection Division

[1] Biosafety is defined as: "Means to regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health." UNEP/CBD. 1992. Convention on Biological Diversity: Article 8(g).

[2] Biosecurity is defined as: "A strategic and integrated approach to analyzing and managing relevant risks to human, animal and plant life and health and associated risks to the environment." FAO. 2007. FAO Biosecurity Toolkit. ISBN 978-92-5-105729-2.

Acronyms

AGN	Nutrition and Consumer Protection Division of the FAO
AGNS	Food Quality and Standards Service of the FAO
AII	Allergy and Immunology Institute of the ILSI
APUA	Alliance for the Prudent Use of Antibiotics
BCIL	Biotechnology Consortium of India Limited
CAC	Codex Alimentarius Commission
CBAC	Canadian Biotechnology Advisory Committee
CBD	Convention on Biological Diversity
CPB	Cartagena Protocol on Biosafety
Defra	United Kingdom Department for Environment, Food and Rural Affairs
DNA	Deoxyribonucleic acid
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia and New Zealand
GC-MS	Gas chromatography coupled to mass spectrometry
GLP	Good laboratory practice
GM	Genetically modified
GMO	Genetically modified organism
HPLC	High-pressure liquid chromatography
HPLC-NMR	Liquid chromatography coupled to nuclear magnetic resonance
ICGEB	International Centre for Genetic Engineering and Biotechnology
IFBC	International Food Biotechnology Council
IFBiC	ILSI International Food Biotechnology Committee
IFIC	International Food Information Council
IHCP	The Institute for Health and Consumer Protection of Director General JRC
ILSI	International Life Sciences Institute
INFOODS	International Food Data Systems Project
ISP	Independent Science Panel
JRC	Joint Research Center
LMO	Living modified organism
NDL	Nutrient Data Laboratory of the USDA
NOEL	No observed (adverse) effect level
OECD	Organisation for Economic Co-operation and Development
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
T-DNA	Transfer-DNA

TFFBT	Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnologies
Ti	Tumour-inducing plasmid
UN	United Nations
UNIDO	United Nations Industrial Development Organization
USDA	United States Department of Agriculture
US FDA	United States Food and Drug Administration
US NAS	United States National Academy of Sciences
VAD	Vitamin A deficiency
WHO	World Health Organization

Part One

Principles of safety assessment of food derived from recombinant-DNA plants

1. Introduction

Scope of the training package

This package was developed in this context to present a framework for the safety assessment of foods derived from recombinant-DNA plants, based on internationally accepted principles and guidance. Additionally, it introduces other issues related to the topic and provides links to useful resources. Practical information about organizing and delivering a training workshop is also included.

Several international documents are being prepared on safety assessment of genetically modified (GM) foods other than those derived from recombinant-DNA plants, and additional training materials will also be developed by FAO. This particular training package does not address the safety assessment of foods derived from other recombinant organisms (such as microorganisms and animals) or livestock feeds derived from recombinant-DNA plants, nor does it consider the ethical and socio-economic issues, and environmental risks, that may be associated with the release of recombinant-DNA plants.

Objectives

In order to support capacity building in food safety assessment, FAO, in collaboration with many international, intergovernmental and governmental bodies, has supported the development of a standardized training programme to assist countries in implementing international documents related to the risk analysis of products containing or derived from genetically modified organisms. Specifically, the training package should be used for implementation of programmes that:

- promote a harmonized international regulatory approach to countries that have requested such guidance, to ensure consistency and uniformity in the application of international standards;
- provide regulators in the beneficiary countries with information on internationally accepted approaches to the evaluation of foods derived from recombinant-DNA plants;
- endorse a transparent, science-based approach to the safe introduction and use of foods derived from recombinant-DNA plants.

Target audience and trainer qualifications

The target audience includes national food safety regulators, authorities, and/or scientists tasked with training others to undertake the safety assessment of foods derived from recombinant-DNA plants. While developed mainly for government agencies in developing countries, this tool may also be of use to agencies in developed countries, as well as to donor organizations and agencies supporting capacity building activities in food safety.

Expected qualifications for the trainer include a Ph.D. degree in natural sciences or an equivalent combination of education and experience, and extensive experience as a regulator or as a senior scientist active in a scientific area relevant to the safety assessment of GM foods. Examples of relevant areas include: molecular biology, plant breeding, biochemistry,

immunology, toxicology, and human or livestock health and nutrition. Experience with working in a multidisciplinary environment with people of different nationalities, ethnic and cultural backgrounds would be an asset. Proficiency in using computers, on-line communication and information retrieval is expected. The trainer is also expected to have in-depth knowledge of both public and private sector research and development, and to have excellent language, communication and presentation skills, particularly to different audiences. A publication record in the scientific literature or in dossier evaluation is required. Trainers should be selected on their personal capacities in a transparent manner. For international training courses attention should be paid to geographical and gender balance.

Contents of the training package

The package is composed of three parts with a CD-ROM containing the visual aids and other relevant reference materials. The first part, *Principles of safety assessment of foods derived from recombinant-DNA plants,* provides guidance text for the implementation of an effective framework for safety assessment of foods derived from recombinant-DNA plants. The second part, *Tools and techniques for trainers*, offers a practical guide for preparing and delivering a workshop on the topic of safety assessment of foods derived from recombinant-DNA plants. This section includes various checklists and forms, a sample workshop agenda, sample workshop evaluation sheet, and five useful presentation modules for trainers. All forms, presentations and copies of the relevant Codex Alimentarius documents are included in the CD-ROM in electronic format. The third part, *Case studies*, presents three safety assessment dossiers that have been summarized for training purposes[3]. All three case studies have been developed based on the data and information submitted for the food safety assessment regulatory evaluation conducted by Governmental agencies such as Health Canada, the United States Food and Drug Administration, and Food Standards Australia New Zealand. The case studies are in-kind contributions that have been provided by Agbios, Inc., Ottawa, Canada, and the Canadian Government, represented by Health Canada[4].

Expected outcomes

Upon completion of training administered using this training tool as a guide, the audience will be able to plan and deliver GM food safety assessment training for national food safety authorities, regulators, and/or scientists in their own training programmes ●

3 In order to enhance the utility of the case studies for training purposes, certain information has been summarized and the data presented in the case studies are only a subset of those actually submitted. The case studies do not reflect a complete application, nor a complete safety assessment.

4 These case studies are included in this training package without any modification or enhancement by FAO. The views expressed in the case studies do not necessarily reflect the views of FAO.

2. Concepts and principles of safety assessment of food derived from recombinant-DNA plants (within international frameworks)

Introduction

Modern biotechnology broadens the scope of genetic changes that can be introduced into organisms used for food. However, it does not inherently result in foods that are less safe than those produced by more conventional techniques (OECD, 1993; US NAS, 2004). This principle has important ramifications for the safety assessment of GM foods. It means that a new or different standard of safety is not required, and that previously established principles for assessing food safety still apply. Moreover, introducing specific genetic changes should enable a more direct and focused assessment of safety.

While countries may differ in statutory and non-statutory approaches to regulating foods derived from recombinant-DNA plants, the criteria used to assess the safety of these products is generally consistent from one country to another (World Bank, 2003). This reflects the concerted efforts that have been made internationally to harmonize the risk assessment of foods derived from modern biotechnology (Table 2.1). The outcomes of these consultations have contributed significantly to the development of internationally accepted approaches to assessing the safety of foods derived from biotechnology, as articulated in two important documents published in 2003 by the Codex Alimentarius Commission (CAC)[5]: *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* (hereinafter referred to as "Codex Principles"; see Appendix 1) and *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants CAC GL 45-2003* (hereinafter referred to as "Codex Guideline"; see Appendix 2).

These documents acknowledge the inadequacy of applying already established risk assessment principles to foods, which by nature are complex compounds and not single chemicals that can be investigated individually. Nevertheless, the documents describe the safety assessment of foods derived from recombinant-DNA plants as a process within the established framework of risk assessment. Safety assessment is in essence the first step in identifying any hazards that may be associated with the food, after which the risks to human health are evaluated.

Role of Codex Alimentarius Commission (CAC) in setting food safety standards

The CAC was created in 1963 by FAO and the World Health Organization (WHO) to develop food standards, guidelines and related texts such as codes of practice under the Joint FAO/WHO Food Standards Programme. The main purposes of this programme are protection of the health of consumers and ensuring fair trade practices in the food trade, and promoting harmonization of all food standards work undertaken by international governmental and non-governmental organizations[6]. The 23rd Session of the CAC agreed to establish the *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology (TFFBT) under the following Terms of Reference:

- to elaborate standards, guidelines or other principles, as appropriate, for foods derived from biotechnology;

[5] At the same time, the Codex Alimentarius Commission also published a third document, *Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms.*

[6] http://www.codexalimentarius.net/web/index_en.jsp

- to coordinate and closely collaborate, as necessary, with appropriate Codex Committees within their mandate as related to foods derived from biotechnology;
- to take full account of existing work carried out by national authorities, FAO, WHO, other international organizations and other relevant international fora.

The Task Force successfully completed its work within the original four-year time frame, culminating with the publication of the Codex Principles and Guideline.

Table 2.1. Some key international consultations addressing the safety assessment of foods derived from recombinant-DNA plants (1990-2006)

Year	*Organization*	*Title and link (where available)*
1990	FAO/WHO	Strategies for assessing the safety of foods produced by biotechnology, a joint FAO/WHO consultation. Geneva, Switzerland, 5–10 Nov. 1990. (http://www.who.int/foodsafety/publications/biotech/1990/en/index.html)
1990	IFBC	Biotechnologies and food: assuring the safety of foods produced by genetic modification. Regulatory Toxicology and Pharmacology, 12: S1–S196.
1993	WHO	Health aspects of marker genes in genetically modified plants. Report of a WHO Workshop. Copenhagen, Denmark, 21–24 Sept. 1993.
1994	WHO	Application of the principles of substantial equivalence to the safety evaluation of foods or food components from plants derived by modern biotechnology. Report of a WHO Workshop, Copenhagen, Denmark, 31 Oct.–4 Nov. 1994.
1996	FAO/WHO	Biotechnology and food safety. Report of a Joint FAO/WHO Consultation, Rome, Italy, 30 Sept.–4 Oct. 1996. FAO Food and Nutrition Paper No. 61.
1996	ILSI	ILSI Allergy and Immunology Institute (AII) guidance for assessing the allergenic potential of foods derived from biotechnology.
1997	OECD	Safety assessment of new foods: results of an OECD survey of serum banks for allergenicity testing, and use of databases. (http://www.olis.oecd.org/olis/1997doc.nsf/LinkTo/sg-icgb(97)1-final)
1998	OECD	Report of the OECD workshop on the toxicological and nutritional testing of novel foods. (http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/sg-icgb(98)1-final)
2000	FAO/WHO	Report of a Joint FAO/WHO Expert Consultation on foods derived from biotechnology – safety aspects of genetically modified foods of plant origin. WHO Headquarters, Geneva, Switzerland, 29 May–2 June 2000. (http://www.fao.org/ag/agn/agns/biotechnology_expert_2000_en.asp)
2000	CAC	First session of the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology. Chiba, Japan, Mar. 2000. (http://www.who.int/foodsafety/publications/biotech/ctf_march2000/en/index.html)
2001	FAO/WHO	Allergenicity of genetically modified foods, a joint FAO/WHO consultation on foods derived from biotechnology. Rome, Italy, 22–25 January 2001.
2001	CAC	Second session of the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology. Chiba, Japan, Mar. 2001. (http://www.who.int/foodsafety/publications/biotech/ctf_march2001/en/index.html)
2002	OECD	Report of the OECD Workshop on the nutritional assessment of novel foods and feeds. (http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/env-jm-mono(2002)6)
2002	CAC	Third session of the Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology. Yokohama, Japan, March 2002. (http://www.who.int/foodsafety/publications/biotech/ctf_march2002/en/index.html)
2002	WHO	The stakeholders' meeting on WHO draft document "WHO – modern food biotechnology, human health and development: an evidence-based study". WHO, Geneva.
2003	CAC	Fourth session of the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology. Yokohama, Japan, March 2003. (http://www.who.int/foodsafety/publications/biotech/july2003/en/index.html)
2003	OECD	Report on the questionnaire on biomarkers, research on the safety of novel foods and feasibility of post-market monitoring. (http://www.olis.oecd.org/olis/2003doc.nsf/LinkTo/env-jm-mono(2003)9)
2006	FAO	FAO expert consultation on biosafety within a biosecurity framework: Contributing to sustainable agriculture and food production. 28 February–3 March 2006, Rome, Italy. (http://www.fao.org/ag/agn/agns/meetings_consultations_2006_en.asp)

List of international consultations on food safety

Several international organizations have identified the need to convene experts in order to address the scientific and other issues raised regarding the safety aspects of foods derived from recombinant-DNA plants or the consequence of their release into the environment, to rationalize the large number of discussions taking place on the topic in different countries to which these products are being targeted. Organizations such as FAO, WHO, OECD, ILSI and IFBC played an important role in the 1990s by facilitating and supporting several expert consultations on the subject, which were followed by the establishment of the Codex Alimentarius Commission in 2000. The major references are listed in the Table 2.1 ●

3. The comparative approach for safety assessment of foods derived from recombinant-DNA plants

Introduction

To date, the safety assessment of foods derived from recombinant-DNA plants has been based on the principle that these products can be compared with conventional counterparts that have an established history of safe use. The objective is to determine if the food presents any new or altered hazard in comparison with its conventional counterpart. The goal is not to establish an absolute level of safety, but the food should be as safe as its conventional counterpart in the sense that there is a reasonable certainty that no harm will result from its intended use under the anticipated conditions of processing and consumption.

Principles of the comparative approach

Accounting for processing and consumption patterns is important even for conventional foods. A number of plants consumed by humans are acutely toxic in their raw state, but are accepted as food because processing methods alter or eliminate this toxicity. For example, the cassava root is quite toxic, but proper processing converts it into a nutritious and widely consumed food. Soybeans and lima beans, among other crops, contain antinutrients (e.g. soybean trypsin inhibitor and lectins) and require proper processing. Potatoes and tomatoes can contain toxic levels of the glycoalkaloids solanine and alpha-tomatine, respectively. Thus, the presence of a toxicant in a plant variety does not necessarily eliminate its use as a food source. In considering the safety of the food derived from recombinant-DNA plants, it is therefore important to examine the range of possible toxicants, critical nutrients and other relevant factors, as well as its processing, intended use and exposure levels. The choice of compounds to be analysed is based on experience gained with conventional crops, and the OECD Task Force for the Safety of Novel Foods and Feed has developed a number of internationally agreed Consensus Documents that provide guidance on the particular compounds that should be analysed.

The comparative approach has been embodied in the concept of substantial equivalence – a concept that was developed before foods derived from modern biotechnology came to the market. The concept was first described in an OECD publication in 1993 (OECD, 1993). This document was developed by some 60 experts from 19 OECD countries, who spent more than two years discussing how to assess the safety of foods derived from modern biotechnology. The concept of substantial equivalence was further endorsed by an FAO/WHO Joint Expert Consultation in 1996. This consultation recognized that the establishment of substantial equivalence is not an assessment of safety per se, but that it gives structure to the safety analysis of the characteristics and composition of food derived from recombinant-DNA plants. Establishing equivalence to a conventional food with a history of safe consumption indicates that the new product will be as safe as the conventional food under similar consumption patterns and processing practices.

One important benefit of the concept of substantial equivalence is that it provides flexibility, which can be useful in the safety assessment of food derived from modern biotechnology. It is a

tool that helps to identify any difference, deliberate or unintended, which might be the focus of further safety evaluation. Because it facilitates a comparative process for evaluating safety, the concept of substantial equivalence can be applied at several points along the food chain (e.g. at the level of the harvested or unprocessed food product, the individual processed fractions, or the final food product or ingredient). This allows the safety assessment to be targeted to the most appropriate level based upon the nature of the product under consideration.

The Joint FAO/WHO Expert Consultation on Food Derived from Biotechnology – Safety Aspects of Genetically Modified Foods of Plant Origin (FAO/WHO, 2000) re-examined the concept of substantial equivalence and concluded that the safety assessment requires an integrated stepwise case-by-case approach, which can be aided by a structured series of questions. They reaffirmed that the concept of substantial equivalence, which focuses on the determination of similarities and differences between the foods derived from recombinant-DNA plants and their conventional counterparts and aids in the identification of potential safety and nutritional issues, and that this comparative approach is the most appropriate strategy for evaluating the safety and nutritional quality of foods derived from recombinant-DNA plants. They further clarified that the concept of substantial equivalence is not a safety assessment in itself as it does not characterize hazard; rather it should be used to structure the safety assessment of a food derived from a recombinant-DNA plant relative to its conventional counterpart (the comparator). The consultation was satisfied with the approach used to assess the safety of foods derived from recombinant-DNA plants that have been approved for commercial use. The consultation concluded that the application of the substantial equivalence concept contributes to a robust safety assessment framework. In fact,there are currently no alternative strategies that provide a better assurance of safety (FAO/WHO, 2000).

CODEX GUIDELINE PARAGRAPH 13. The concept of substantial equivalence is a key step in the safety assessment process. However, it is not a safety assessment in itself; rather it represents the starting point which is used to structure the safety assessment of a new food relative to its conventional counterpart[7]. This concept is used to identify similarities and differences between the new food and its conventional counterpart . It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods derived from recombinant-DNA plants. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the new product can be considered relative to its conventional counterpart.

7 The concept of *substantial equivalence* as described in the report of the 2000 joint FAO /WHO expert consultations (Document WHO/SDE/PHE/FOS/00.6, WHO, Geneva, 2000).

The Codex Guideline includes the reference to substantial equivalence (paragraph 13). Note that wherever text from the Codex Guideline is referenced, it is identified by both a box and a reference to the relevant paragraphs of the Guideline (Appendix 2).

Identifying unintended effects

The applicability of the substantial equivalence concept in the safety assessment of recombinant-DNA plants has been questioned (Millstone *et al.*, 1999). However, the utility of the concept is well established, and several expert consultations (FAO/WHO, 1996, 2000) have found that safety assessments based on the concept of substantial equivalence are the most practical approach developed to date to address the safety of foods developed through modern biotechnology. Equivalence can be established relatively easily when the new gene product is targeted and can be utilized directly without resulting in any further modification to the existing metabolic pathways of the plant. However, the changes in recombinant-DNA derived plants and food sometimes may not be reflected in the known compounds that are preselected for equivalence assessment, due to unintended changes resulting from insertion of the new gene. In such cases, non-targeted profiling approaches will be essential to identify any unintended effects that are not predictable. Genomic strategies using bioinformatics tools can be effective in analysing unintended changes occurring at the RNA transcript, amino acid, protein or metabolic levels (Stiekema and Nap, 2004). Paragraphs 14 to 17 of the Codex Guidelines specifically address unintended changes.

CODEX GUIDELINE PARAGRAPH 14. In achieving the objective of conferring a specific target trait (intended effect) to a plant by the insertion of defined DNA sequences, additional traits could, in some cases, be acquired or existing traits could be lost or modified (unintended effects). The potential occurrence of unintended effects is not restricted to the use of in vitro nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in conventional breeding. Unintended effects may be deleterious, beneficial, or neutral with respect to the health of the plant or the safety of foods derived from the plant. Unintended effects in recombinant-DNA plants may also arise through the insertion of DNA sequences and/or they may arise through subsequent conventional breeding of the recombinant-DNA plant. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA plant would have an unexpected, adverse effect on human health.

CODEX GUIDELINE PARAGRAPH 15. Unintended effects can result from the random insertion of DNA sequences into the plant genome which may cause disruption or silencing of existing genes, activation of silent genes, or modifications in the expression of existing genes. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels may give rise to secondary biochemical effects or changes in the regulation of metabolic pathways and/or altered levels of metabolites.

CODEX GUIDELINE PARAGRAPH 16. Unintended effects due to genetic modification may be subdivided into two groups: those that are "predictable" and those that are “unexpected”. Many unintended effects are largely predictable based on knowledge of the inserted trait and its metabolic connections or of the site of insertion. Due to the expanding information on plant genome and the increased specificity in terms of genetic materials introduced through recombinant-DNA techniques compared with other forms of plant breeding, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse potential changes at the level of gene transcription and message translation that could lead to unintended effects.

CODEX GUIDELINE PARAGRAPH 17. The safety assessment of foods derived from recombinant-DNA plants involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information are necessary to assess unintended effects because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, provide assurance that the food is unlikely to have an adverse effect on human health. The assessment for unintended effects takes into account the agronomic/phenotypic characteristics of the plant that are typically observed by breeders in selecting new varieties for commercialization. These observations by breeders provide a first screen for plants that exhibit unintended traits. New varieties that pass this screen are subjected to safety assessment as described in Sections 4 and 5.

Some examples of substantial equivalence tests

As the following examples demonstrate, new products with intentionally altered nutritional profiles will challenge our ability to assess unintended consequences. The first example relates to genetically engineered low-glutelin rice, which has been created by introducing the glutelin-encoding gene in the antisense orientation, for commercial production of sake. The decrease in glutelin level was associated with an unintended increase in the level of prolamins. The change in prolamin level was not detected by standard nutritional analyses, such as total protein and amino acid profiles, but was only observed following sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE). While the change in prolamin level did not affect the industrial application, it could affect nutritional quality and allergenic potential if the rice were used as a food. A second example relates to genetically engineered “Golden Rice” designed to express increased levels of beta-carotene, a precursor to vitamin A. Unexpectedly, it was found that this modification was accompanied by higher levels of xanthophylls, a change that would not have been apparent from standard nutritional analyses but was detected from high-pressure liquid chromatography (HPLC) analyses for carotenoids. As these two examples illustrate, targeting a single nutrient of a complex metabolic pathway can lead to unintended alterations in the levels of other constituents, and specialized analytical methodologies may be required to assess changes in the overall nutrient profile.

Another consequence of the introduction of significant nutritional changes in a food may be the requirement for post-market monitoring of this food. In such cases, the primary objective

would be to determine if the patterns of dietary intake are altered by the introduction of the food to the market.

Substantial equivalence – issues of concern in its application

The substantial equivalence concept is used to structure the safety assessment and to identify similarities and differences between the new food and its conventional counterpart. It is recognized that the substantial equivalence is not a safety assessment in itself, nor is it an endpoint but just a starting point for the safety assessment (FAO/WHO, 2000). The following points should be considered when adopting the substantial equivalence approach.

First, the concept depends on the presence of a relevant comparator and on the information that is available or can be generated for the comparator. The choice of comparator is therefore crucial to effective application of the concept. The comparator must have a well documented history of safe use. If adverse effects have been associated with the particular food type, specific components that are considered to cause these effects should be described and well characterized to permit effective comparison. Establishing a baseline for comparative analyses can be challenging if the recombinant-DNA plant is developed for cultivation under conditions of stress that are non-permissive for growth of the conventional counterpart.

Second, the plant-specific and relevant parameters that should be compared to establish substantial equivalence must be identified on a case-by-case basis because there is a possibility that unintended compositional changes may be overlooked in the comparative approach.

Third, the inherent variability in most parameters measured in biological systems can make interpretation of the significance of observed changes difficult. A comparative approach therefore relies on an accurate understanding of the baseline variation in the parameters to be compared. The choice of comparator will influence the range of the baseline data and must be carefully evaluated in relation to the relevant risk hypothesis that underlies parameter selection.

Final remarks

Safety assessment of a whole food requires a different approach from that which has been used to assess the safety of individual chemical substances such as food additives or pesticides. Unlike individual chemical substances, whole foods are composed of a variety of compounds that contribute to their nutritional value. Foods produced from many crops also contain natural toxicants, antinutrients, and other substances that are important to the plant but which if present in sufficient quantities in the food may be harmful to humans. The Codex Guideline on recombinant-DNA plants recommends that a comparative assessment be used to determine if a food derived from a recombinant-DNA plant is as safe as an appropriate comparator food. The underlying assumption of this approach is that conventionally bred and cultivated crops have gained a history of safe use for consumers, animals and the environment. Using conventional breeding methods, developers have selected varieties of crops that each contain thousands of substances that are considered overall to be safe for human consumption.

References

FAO/WHO. 1996. Biotechnology and food safety, FAO/WHO consultation 30 Sept–4 Oct 1996. Food and Agriculture Organization, Rome and World Health Organization, Geneva. http://www.fao.org/ag/agn/food/pdf/biotechnology.pdf

FAO/WHO. 2000. Safety aspects of genetically modified foods of plant origin, FAO/WHO consultation 29 May–2 June 2000. Food and Agriculture Organization, Rome and World Health Organization, Geneva. http://www.who.int/foodsafety/publications/biotech/ec_june2000/en/index.html

Millstone, *et al.* 1999. Beyond substantial equivalence. *Nature*, 401: 525–526.

OECD. 1993. Safety evaluation of foods derived by modern biotechnology, concepts and principles. Organization for Economic Co-operation and Development (OECD), Paris.

OECD. 2000. Report of the task force for the safety of novel foods and feeds. C(2000)86/ADD1. Organization for Economic Co-operation and Development (OECD), Paris.

Stiekerma W.J. & Nap P.J. 2004. Bioinformatics for biosafety: predicting the allergenicity in GM food. *In* P.J. Nap, A. Atanosov & W.J. Stiekema, eds. *Genomics for biosafety in plant biotechnology*, pp. 98–116. NATO Science Series, Series I – Life and behavioral sciences, Vol 359. Amsterdam, IOS Press.

United States National Academy of Sciences. 2004. Safety of genetically engineered foods: approaches toassessing unintended health effects. Washington, DC, The National Academies Press.

World Bank. 2003. Biosafety regulation: a review of international approaches (Report No. 26028). The World Bank Agriculture and Rural Development Department, Washington, DC.

Additional resources

ILSI. 2004. Nutritional and safety assessment of foods and feeds nutritionally improved through biotechnology. *Comp. Rev. Food Sci. Food Safety*, 3: 38–104.

OECD. 2000. Genetically modified foods: widening the debate on health and safety. (updated document of "Substantial equivalence and the safety assessment of GM foods) Organization for Economic Co-operation and Development, Paris. http://www.oecd.org/dataoecd/34/30/2097312.pdf

WHO. 1995. Application of the principles of substantial equivalence to safety evaluation of foods or food components from plants derived by modern biotechnology. Report of a WHO Workshop. World Health Organization, Geneva. WHO/FNU/FOS/95.1.

WHO. 2005. Modern food biotechnology, human health and development: an evidence-based study. World Health Organization, Geneva. http://www.who.int/foodsafety/publications/biotech/biotech_en.pdf ●

4. The framework for the safety assessment of foods derived from recombinant-DNA plants

Introduction

Recombinant-DNA plants developed for food purposes have undergone safety assessment procedures, as required by various national regulatory systems, since the early 1990s. The frameworks used to structure the safety assessments have been continually developed by international organizations and standard-setting bodies to ensure the safety of products and to promote trade through harmonized regulations. The concept of substantial equivalence was introduced by OECD in 1993 as a feasible way of structuring the safety assessment of recombinant-DNA plants (OECD, 1993). The concept was later adopted by the WHO and FAO as a useful starting point for the safety assessment of recombinant-DNA plants, and now represents an essential component of all regulatory frameworks on a global scale. The rationale behind the concept's utility and adoption is that recombinant-DNA plants developed for food purposes are considered to be essentially equivalent (chemically) to their conventional counterparts, with the exception of the few defined changes that have been introduced.

Extensive general biological characterization and toxicological testing are not therefore thought to be necessary because the comparative approach should reveal relevant biological differences. Safety assessment of recombinant-DNA plants developed for food purposes is nevertheless often based on additional extensive data collected on the immunological and toxicological properties of the new plant variety. The current framework of safety assessment is thus based on both the structured comparative basis enshrined in the concept of substantial equivalence and additional analyses of the toxicological and immunological properties of the intentional and potential unintentional effects of the introduced genetic modifications. The goal of the safety assessment of foods derived from recombinant-DNA plants is to examine the intentional and unintentional consequences of the specific modification on the food components and to establish a comparative safety level by drawing on the history of safe use of the conventional plant counterpart.

The Codex framework of the safety assessment

Based on the Codex "Principles for the Risk Analysis of Foods Derived from Modern Biotechnology" (2003), the Codex "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants" was introduced in 2003. This training tool provides a detailed introduction to the conduct of food safety assessment based on the Codex framework for the safety assessment of GM foods (CAC/GL45-2003). The stepwise approach to the safety assessment described in the Codex Guideline is presented with reference to Codex guideline paragraphs 18–21.

CODEX GUIDELINE PARAGRAPH 18.
The safety assessment of a food derived from a recombinant-DNA plant follows a stepwise process of addressing relevant factors that include:

A) Description of the recombinant-DNA plant;
B) Description of the host plant and its use as food;
C) Description of the donor organism(s);
D) Description of the genetic modification(s);
E) Characterization of the genetic modification(s);
F) Safety assessment:
   a) expressed substances (non-nucleic acid substances);
   b) compositional analyses of key components;
   c) evaluation of metabolites;
   d) food processing;
   e) nutritional modification; and
G) Other considerations.

CODEX GUIDELINE PARAGRAPH 19. In certain cases, the characteristics of the product may necessitate development of additional data and information to address issues that are unique to the product under review.

CODEX GUIDELINE PARAGRAPH 20. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, Good Laboratory Practice. Primary data should be made available to regulatory authorities at request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

CODEX GUIDELINE PARAGRAPH 21. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. The expected endpoint of such an assessment will be a conclusion regarding whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. In essence, therefore, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed and if so to make well-informed and appropriate decisions.

The specific data requirements in the Codex Guideline for describing the features of recombinant-DNA plants are outlined in paragraphs 22–33, and are explained in further detail in the following sections.

Description of the recombinant-DNA plant

A recombinant-DNA plant is produced as a result of successful gene transfer (transformation) followed by stable integration of the recombinant-DNA (transgene) into the nuclear chromosome(s) or organelle genome(s) of the plant. The biotechnologist uses classical plant breeding techniques such as selfing to make this initial plant homozygous at the recombinant locus (loci). The recombinant-DNA can then be stably transferred through generations without segregation. The name of the progeny of such a recombinant-DNA plant is also defined by and refers to the initially produced recombinant-DNA plant. Each plant lineage produced from a successful transfer, plant regeneration and propagation is called an "event" or a "case".

It is important for the safety assessor to understand the recombinant-DNA plant to be evaluated. For example, a clear understanding of the term "event" is essential to the application of a "case-by-case" safety assessment. Because each "event" represents a unique insertion site (or sites) of the recombinant-DNA (transgene), the resulting phenotypic properties of the regenerated recombinant plants are likely to differ. Thus, whereas the general biological properties of the recombinant-DNA will be similar across different insertion "events", potential unintentional effects on the host genome may vary because the insertions may cause different effects depending on their location and insertion number (see Box 4.1). An "event" may represent a plant with a single insert, or with multiple inserts transferred at the same time. For example, a single event may comprise several insertions of recombinant-DNA that encode both insecticide resistance and herbicide resistance, if these traits were transferred at the same time.

CODEX GUIDELINE PARAGRAPH 22. A description of the recombinant-DNA plant being presented for safety assessment should be provided. This description should identify the crop, the transformation event(s) to be reviewed and the type and purpose of the modification. This description should be sufficient to aid in understanding the nature of the food being submitted for safety assessment.

Plants containing recombinant-DNA from independent transfer events have "stacked" traits, and are often produced by crossing plant cultivars that each carry unique and well characterized "events". In this way, more recombinant-DNA insertions (and "events") that have been selected based on good performance in their original recipient host can be assembled in a single new plant variety. Plants with stacked recombinant-DNA insertions (transgenes) are also evaluated for potential interactions occurring between the DNA insertions, as a part of the safety assessment.

The first two to three pages of the example dossier extracts provided with this tool contain relevant descriptive information to provide the safety assessor with the key characteristics and intended purpose of the recombinant-DNA plant.

Description of the host plant and its use as food

Paragraphs 23–25 request information on the host plant and its known uses for food. A thorough knowledge of the non-modified host plant is necessary to apply the concept of substantial equivalence as a starting point for establishing safety. In the case of food safety assessment, this descriptive knowledge is critical for identifying the natural range and variation of key nutritional components, and of known toxicants (e.g. alkaloids in potatoes and tomatoes, curcurbiticin in squash and zucchini), antinutrients and potential allergens. These compounds and their respective concentrations will vary between crops, cultivars and growth conditions in a similar way to those of conventional varieties.

Natural variations in such compounds are known as and described by the "baseline level". Efforts are underway to establish databases that contain descriptive data on the range of baseline levels for key chemical compounds naturally present in crop plants. Crop plants naturally contain several thousand chemical compounds, of which many will cause undesired effects in toxicological tests if extracted singly and administered in high doses to experimental animals. It is therefore challenging to evaluate the biological effects potentially caused by minor variations or fluctuations in the levels of a particular plant compound. Therefore, knowledge of the natural variation in the baseline level of key compounds in conventional varieties of the plant is of great use in the safety assessment of complex data sets obtained from chemical analysis of recombinant-DNA plants.

Post-harvest processing of plant components may also alter the levels of particular plant compounds that are of nutritional value. Hence knowledge of the use, processing and consumption, as well as the properties, of the final product of the conventional food crop is important in establishing a sound basis for appropriate comparison with the foods derived from recombinant-DNA plants. Such information is provided in the example documents/dossiers.

An information source that provides extensive information on host plant biology is the OECD Consensus Documents. These consensus documents comprise technical information for use during the regulatory assessment of products of biotechnology. They focus on the biology of organisms (such as plants, trees or micro-organisms) or the introduced traits and can be accessed at: http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html

CODEX GUIDELINE PARAGRAPH 23. A comprehensive description of the host plant should be provided. The necessary data and information should include, but need not be restricted to:

A) common or usual name; scientific name; and taxonomic classification;
B) history of cultivation and development through breeding, in particular identifying traits that may adversely impact on human health;
C) information on the host plant's genotype and phenotype relevant to its safety, including any known toxicity or allergenicity; and
D) history of safe use for consumption as food.

CODEX GUIDELINE PARAGRAPH 24. Relevant phenotypic information should be provided not only for the host plant, but also for related species and for plants that have made or may make a significant contribution to the genetic background of the host plant.

CODEX GUIDELINE PARAGRAPH 25. The history of use may include information on how the plant is typically cultivated, transported and stored, whether special processing is required to make the plant safe to eat, and the plant's normal role in the diet (e.g. which part of the plant is used as a food source, whether its consumption is important in particular subgroups of the population, what important macro- or micro-nutrients it contributes to the diet).

Description of the donor organism(s)

Information about the natural history of the donor organism for the recombinant-DNA sequences is required, particularly if the donor or other members of

CODEX GUIDELINE PARAGRAPH 26. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health (e.g. presence of anti-nutrients). The description of the donor organism(s) should include:

A) its usual or common name;
B) scientific name;
C) taxonomic classification;
D) information about the natural history as concerns food safety;
E) information on naturally occurring toxins, anti-nutrients and allergens; for microorganisms, additional information on pathogenicity and the relationship to known pathogens; and
F) information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g. possible presence as contaminants).

its genus normally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. If the donor organism contains known allergens particular caution must be exercised (Codex Guideline paragraph 26). When the food derived from recombinant-DNA plants contains genes from such sources, it is assumed that the novel gene product is allergenic unless proven otherwise. The assessment of allergenicity takes this aspect into account. In cases where the recombinant-DNA originates from sources with no history of allergenicity, the current approach to assessing allergenicity or toxicity relies primarily upon amino acid sequence comparisons and the stability of the novel protein to digestion and processing. Notably, this latter comparison is not made with respect to the conventional counterpart, but draws on a broad knowledge base regarding the biological properties of known allergens in food.

Currently, most commercially used DNA sequences inserted into recombinant-DNA plants are collected from commonly occurring soil bacteria and pathogenic plant bacteria and viruses, and hence they often have a known history in agriculture. Establishing prior human exposure to the recombinant-DNA source is useful as a starting point to identify possible toxic and allergenic properties of the gene products. Nevertheless, care should be taken in drawing safety inferences from such information, given the potentially altered expression levels, cellular locations and exposure routes of the recombinant-DNA derived proteins. Information is provided on the donor sources in the example documents/dossiers.

The OECD Consensus Documents also provide information on the biology of gene donors: http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html

Description of the genetic modification(s)

The data requirements related to the genetic modifications serve two purposes: (i) to allow a detailed understanding of the resulting genetic insertions and their locations in the host plant; (ii) to allow unique identifiers to be constructed based on the event-specific insertion sites of the recombinant-DNA in the plant host genome (Codex Guideline paragraph 27). The latter information can be important both for the developer of a recombinant-DNA plant, as a means to ensure commercial distribution and use, and for some countries with mandatory food labelling requirements, to allow event-specific monitoring of recombinant-DNA in the food chain. For the biological safety assessment, it is important to have information on DNA insertion numbers and sites in order to evaluate the effect of the insertions on the host plant genome and to predict potential phenotypic changes. A detailed description of the molecular characteristics of the recombinant-DNA plant is required in order to demonstrate that the developer has critically analysed the plant and its products, including all introduced genes and expressed proteins. It should be noted that the recombinant-DNA plants have undergone extensive selective breeding subsequent to the initial gene transfer event and prior to seeking regulatory approval. Thus, the developer is likely to provide a range of data in the application dossier to demonstrate that the recombinant-DNA plant expresses only the intended phenotypic changes. As seen from the example documents/dossiers, extensive information on the characterization of the genetic modifications is provided.

The method by which the novel traits are introduced into the host plant determines, in part, the information required for the safety assessment of the genetic properties of the plant (Codex Guideline paragraph 28–29). The two principal methods for introducing new genetic

CODEX GUIDELINE PARAGRAPH 27. Sufficient information should be provided on the genetic modification to allow for the identification of all genetic material potentially delivered to the host plant and to provide the necessary information for the analysis of the data supporting the characterization of the DNA inserted in the plant.

CODEX GUIDELINE PARAGRAPH 28. The description of the transformation process should include:
A) information on the specific method used for the transformation (e.g. *Agrobacterium*-mediated transformation);
B) information, if applicable, on the DNA used to modify the plant (e.g. helper plasmids), including the source (e.g. plant, microbial, viral, synthetic), identity and expected function in the plant; and
C) intermediate host organisms including the organisms (e.g. bacteria) used to produce or process DNA for transformation of the host organism.

CODEX GUIDELINE PARAGRAPH 29. Information should be provided on the DNA to be introduced, including:
A) the characterization of all the genetic components including marker genes, regulatory and other elements affecting the function of the DNA;
B) the size and identity;
C) the location and orientation of the sequence in the final vector/construct; and
D) the function.

material into plant cells are (i) *Agrobacterium*-mediated transformation and (ii) microprojectile bombardment.

(i) *Agrobacterium*-mediated gene transfer. *Agrobacterium tumefaciens* is a soil-borne phytopathogen that naturally uses genetic transformation processes to subvert the metabolic machinery of the host plant cell. It does so to divert some of the host's organic carbon and nitrogen supplies to the production of nutrients (opines) that can be specifically catabolized by the invading bacteria. Parasitized cells are also induced to proliferate. Crown gall tumour disease is a direct result of the incorporation of a region of transfer-DNA (T-DNA) from a large (150–250 kb) circular Ti (tumour-inducing) plasmid, carried by *A. tumefaciens*, into the host plant genome. An understanding of this natural transformation process, together with the realization that any foreign DNA placed between the T-DNA border sequences can be transferred to plant cells, led to the construction of the first vector and bacterial strain systems for plant transformation (for a review see Hooykaas and Schilperoort, 1992). Since the first record of a transgenic tobacco plant expressing foreign genes, great progress has been made in understanding *Agrobacterium*-mediated gene transfer at the molecular level. *Agrobacterium tumefaciens* naturally infects only dicotyledonous plants, although methods for *Agrobacterium*-mediated gene transfer into monocotyledonous plants have now been developed for rice (Hiei *et al.*, 1994; Cheng *et al.*, 1998), banana (May *et al.*, 1995), maize (Ishida *et al.*, 1996), wheat (Cheng *et al.*, 1997) and sugarcane (Arencibia *et al.*, 1998; Enríquez-Obregón *et al.*, 1998). A thorough analysis of the strategies for practical application of this method has been published (Birch, 1997). *Agrobacterium*-mediated transformation of plant tissue generally results in a low copy number DNA insertion, small numbers of rearrangements, and higher transformation efficiency than direct DNA delivery techniques such as microprojectile bombardment (Powlowski and Somers, 1996; Gelvin, 1998).

(ii) Microprojectile bombardment-mediated gene transfer. Microprojectile bombardment (also known as microparticle bombardment and biolistic transformation) is a technique used to deliver DNA directly to the host genome, and has proven to be useful for the transformation of plant tissues recalcitrant to *Agrobacterium* infection. In short, plasmid or linearized DNA containing the gene(s) of interest is fixed to tungsten or gold particles (microcarriers), which are delivered to host cells at high speed so as to penetrate the plant cells. In the cell, the DNA may separate from the microcarrier and become integrated into the host genome. Microprojectile bombardment can be used to transform tissue explants of most plant species as long as the transformed plant tissue can be regenerated to produce whole plants. As seen from the example documents/dossiers, details on the gene transfer method used and a molecular analysis of the resulting DNA insertion are provided as a standard part of the application for regulatory approval/notification.

Box 4.1. Mechanistic aspects of the transformation process relevant to safety assessment of recombinant-DNA plants

Length and copy numbers of DNA transferred.
It was assumed until 1995 that in *Agrobacterium*-mediated gene transfer the sequences between the left and right borders of the T-DNA were the only transgenic elements transferred to the recipient host. However, Ramanathan and Veluthambi (1995), Wenck *et al.* (1997) and Kononov *et al.* (1997) all demonstrated that plasmid backbone sequences beyond the borders of the T-DNA could be integrated together with the genes of interest. Experiments by Kononov *et al.* (1997) demonstrated that plasmid backbone sequences could be integrated into the host genome coupled with either the right or left border sequences, or as an independent unit unlinked from the T-DNA. The T-DNA can also integrate into the host genome in patterns other than as a single copy at a single site. Multiple copies in direct or inverted repeats and other complex patterns may also occur. The presence of multimeric T-DNA inserts, especially inverted repeat structures, is linked to the phenomenon of transgene silencing (Gelvin, 1998).

In particle bombardment-mediated gene transfer, the transgene integration pattern varies from the full-length introduced transgene to transgene rearrangements that differ in size from the full length insert, occasional concatenation of introduced plasmids carrying the transgene, and variation in copy number among the full-length and partial transgenic elements (Powlowski and Somers, 1996). The copy number of transgene insertions varies from 1 to 20 or more, in addition to the insertion of partial transgene fragments. Multiple copies usually cosegregate as a transgenic locus, indicating that the sequences are either integrated into tightly linked loci or into a single locus, rather than randomly integrated throughout all chromosomes (Powlowski and Somers, 1996). Molecular characterization of transgenic plants produced through microparticle bombardment has provided evidence of extensive rearrangements of transgenic sequences (Powlowski and Somers, 1996). These rearrangements may be observed in Southern blot analyses as hybridizing fragments of a different size to the full-length DNA insert. Larger fragments are indicative of concatenation (head to head or head to tail)[8]. Larger than full-length fragments of transgenic DNA may also be caused by interspersion of inserted DNA with host DNA. For instance, Powlowski and Somers (1998) reported that each of thirteen transgenic oat lines transformed using microparticle bombardment had intact copies of the transgene, as well as multiple, rearranged, and/or truncated transgene fragments. The number of insertion sites varied from 2 to 12, and all fragments of the transgenic DNA cosegregated. The authors demonstrated that the transgenic DNA was interspersed with host DNA. This phenomenon has also been reported for rice (Cooley *et al.*, 1995).

Variation in gene expression levels based on insertion site.
For both gene transfer methods, plants transformed independently with the same plasmid will commonly have different levels of expression, a phenomenon that is not always correlated with copy number (Gelvin, 1998). Instead, differential expression of transgenes has been attributed by some to positional effects, in which the position of the DNA integration site in the host genome affects the level of transgene expression. However, other research has indicated that factors in addition to, or other than, the position of the site of integration also contribute to the level of transgene expression (Gelvin, 1998). This may be caused by the variable arrangements that transgene sequences may have in the host genome. Variable expression of transgenes, or gene silencing,[9] is a documented phenomenon in transgenic plants.

Due to commercial business information claims, the exact technical and practical laboratory details of the recombinant-DNA transfer protocols are rarely provided in the application dossier. Some of the general mechanistic aspects of the transformation process that are relevant to safety assessment of the generated recombinant-DNA plants are explained in more detail in Box 4.1.

References

Arencibia, A.D., Carmona, E.R., Tellez, P., Chan, M.T., Yu, S.M., Trujillo, L.E., & Oramas, P. 1998. An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens*. *Transgenic Res.* 7: 1–10.

Birch, R.G. 1997. Plant transformation: problems and strategies for practical application. Ann. Rev. Plant Physiol. Plant Mol. Biol. 48: 297–326.

[8] Concatemers of the DNA insert may be detected by extensive Southern blot analysis involving digestion of the genomic DNA with a restriction enzyme that cuts at a single site within the transgenic element; multiple copies of the DNA insert will then be resolved by Southern blot analysis. Concatemers may be formed by homologous recombination of the transformed DNA or by blunt end ligation of cohesive ends produced by limited exonuclease activity. Smaller than full-length fragments are evidence of deletions and truncations.

[9] Gene silencing can result from interactions between multiple copies of transgenes and related endogenous genes and is associated with homology-based mechanisms that act at either the transcriptional or post-transcriptional level (Matzke and Matzke, 1998). Silencing that results from the impairment of transcription initiation is often associated with cytosine methylation and/or chromatin condensation (Fagard and Vaucheret, 2000) while post-transcriptional silencing (cosuppression) involves enhanced RNA turnover in the cytoplasm (Matzke and Matzke, 1998). A third category of silencing has also been proposed for the consequences of positional effects, in which flanking plant DNA and/or unfavourable chromosomal location exert a silencing effect on the transgene (Matzke and Matzke, 1998). According to Matzke and Matzke (1998), this type of silencing reflects the epigenetic state of host sequences flanking the insertion site or the tolerance of particular chromosome regions to insertion of foreign DNA.

Cheng, M., Fry, J.E., Pang, S.Z., Zhou, H.P., Hironaka, C.M., Duncan, D.R., Conner, W. & Wan, Y.C. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens. Plant Physiol.* 115: 971–980.

Cheng, X.Y., Sardana, R., Kaplan, H. & Altosaar, I. 1998. Agrobacterium-transformed rice expressing synthetic cry1Ab and cry1Ac genes are highly toxic to striped stem borer and yellow stem borer. *Proc. Nat. Acad. Sci.* USA. 95: 2767–2772.

Cooley, J., Ford, T. & Christou, P. 1995. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. *Theor. Appl. Genet.* 90: 97–104.

Enríquez-Obregón, G.A., Vázquez-Padrón, R.I., Prieto-Sansonov, D.L., de la Riva, G.A. & Selman-Housein, G. 1998. Herbicide resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium-mediated transformation. Planta* 206: 20–27.

Fagard, M. & Vaucheret, H. 2000. (Trans)gene silencing in plants: how many mechanisms? *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 51: 167–194.

Gelvin, S.B. 1998. The introduction and expression of transgenes in plants. *Curr. Opinion Biotechnol.* 9: 227–232

Hiei, Y., Ohta, S., Komari, T. & Kumashiro, T. 1994. Efficient transformation of rice (*Oriza sativa*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6: 271–282.

Hooykaas, P.J.J. & Schilperoort, R.A. 1992. *Agrobacterium* and plant genetic engineering. *Plant Mol. Biol.* 19: 15–38.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. & Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens. Nature Biotechnol.* 4: 745–750.

Kononov, M.E., Bassuner, B. & Gelvin, S.B. 1997. Integration of T-DNA binary vector "backbone" sequences into the tobacco genome: evidence for multiple complex patterns of integration. *Plant J.* 11: 945–957.

Matzke, A.J.M. & Matzke, M.A. 1998. Position effects and epigenetic silencing of plant transgenes. *Curr. Opinion Plant Biol.* 1: 142–148.

May, G.D., Afza, R., Mason, H.S., Wiecko, A., Novak, F.J. & Arntzen, C.J. 1995. Generationof transgenic Banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Biotechnol.*13: 486–492.

OECD. 1993. Safety evaluation of foods derived by modern biotechnology, concepts and principles. Organization for Economic Co-operation and Development (OECD), Paris.

Powlowski, W.P. & Somers, D.A. 1996. Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Mol. Biotechnol.* 6: 17–30.

Powlowski, W.P. & Somers, D.A. 1998. Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. *Proc. Nat. Acad. Sci.* USA 95: 12106–12110.

Ramanathan, V. & Veluthambi, K. 1995. Transfer of non-T-DNA portions of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of TL-DNA. *Plant Mol. Biol.* 28: 1149–1154.

Wenck, A., Czako, M., Kanevski, I. & Marton, L. 1997. Frequent colinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. *Plant Mol. Biol.* 34: 913–922 ●

5. Characterization of the genetic modification(s)

Molecular analysis of the recombinant-DNA insert

Characterization of a recombinant-DNA plant at the molecular level is performed to provide information about the composition and integrity of the inserted DNA, the number and genomic location of the single or multiple sites of insertion, and the level of expression of the introduced protein(s) over time and in different tissues and environments.

As explained in the Section 4, the process of recombinant-DNA plant production may result in a transformed plant that contains a single insert or multiple inserts present in one or several locations in the host plant genome.

Regulatory authorities examine the information on the integrity and copy number of the inserted DNA in recombinant-DNA plants. Biotechnologists usually seek to minimize the copy number and size of the inserted DNA in recombinant-DNA plants to ease the regulatory process by producing fewer genetic changes that require assessment. However, recombinant-DNA plants containing multiple copies of the inserted DNA are not necessarily less "safe" than comparable plants containing only a single copy[10].

CODEX GUIDELINE PARAGRAPH 30. In order to provide clear understanding of the impact on the composition and safety of foods derived from recombinant-DNA plants, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out.

CODEX GUIDELINE PARAGRAPH 31. Information should be provided on the DNA insertions into the plant genome; this should include:

A) the characterization and description of the inserted genetic materials;
B) the number of insertion sites;
C) the organisation of the inserted genetic material at each insertion site including copy number and sequence data of the inserted material and of the surrounding region, sufficient to identify any substances expressed as a consequence of the inserted material, or, where more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food; and
D) identification of any open reading frames within the inserted DNA or created by the insertions with contiguous plant genomic DNA including those that could result in fusion proteins.

Knowledge of the genomic locations in which the transgene(s) have been inserted in the plant genome is necessary to assess if existing genes or regulatory sequences have been affected by the insertion, which may result in altered gene expression patterns and, hence, plant phenotype. To assess whether new protein molecules could be produced from the integration of inserted DNA, DNA sequence-based bioinformatics analyses are used to determine the presence of open reading frames (ORFs) in and around the DNA insert.

An open reading frame is a part of a gene that is transcribed to produce RNA. Bioinformatics analysis is usually focused on both the newly introduced ORFs present in the DNA insert itself and the potential presence or creation of new ORFs produced from the random insertion of DNA into existing ORFs in the plant genome.

A detailed molecular characterization of the recombinant-DNA may be able to address issues related to possible positional effects that lead to variable gene expression, multiple character changes (pleiotropic

[10] One example of an "event" containing a high transgene copy number is in a line of canola (Brassica napus; event 23-198, 23-18-17) approved by the Canadian Government, which was developed by introducing a thioesterase encoding gene from the California bay tree (Laurus nobilis) to increase levels of lauric acid (12:0) and, to a lesser extent, myristic acid (14:0). The original transformation event 23 was estimated to contain 15 copies of the gene, at five independent genetic loci, as shown by Southern blot and segregation analyses.

effects) arising from the DNA insertion, or gene silencing resulting from overexpression of the inserted DNA. However, in the absence of other empirical data, such molecular analyses are unlikely to predict unforeseen effects on the concentrations of key nutrients, antinutrients or endogenous toxins. Thus, additional compositional analyses are preformed.

Where the result of the modification is the expression of a novel protein, the plant material is characterized with respect to the biochemical composition and functionality of the new gene product(s). Several methods are used to verify and measure the expression of the introduced traits in a recombinant-DNA plant. For novel protein-derived traits, serological techniques are frequently used. Such techniques (e.g. Western immunoblotting or enzyme-linked immunosorbent assay [ELISA]) are used to identify the presence of the transgene product and to quantify its level in the sampled material. If the newly inserted trait is one that does not result in the expression of a new or modified protein[11] but, for instance, results in antisense RNA sequences, other techniques (e.g. Northern blotting) are used to measure transcript production.

CODEX GUIDELINE PARAGRAPH 32. Information should be provided on any expressed substances in the recombinant-DNA plant; this should include:

A) the gene product(s) (e.g. a protein or an untranslated RNA);
B) the gene product(s)' function;
C) the phenotypic description of the new trait(s);
D) the level and site of expression in the plant of the expressed gene product(s), and the levels of its metabolites in the plant, particularly in the edible portions; and
E) where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous mRNA or protein.

In addition to the direct biochemical characterization of the inserted trait, regulatory authorities usually assess studies of the recombinant-DNA plant grown under various conditions. Such studies can show that the intended trait is expressed at the desired life stage of the plant cultivar, and that expression is as expected and is stable over environments and plant generations.

The overall concentration of novel proteins expressed in recombinant-DNA plant tissues is low, often less than 0.1 percent on a dry weight basis. Biosafety studies, such as acute toxicity testing (chapter 6), that require relatively large amounts of material are often not feasible using the protein purified from plant tissue. Instead, these studies normally make use of protein purified from bacterial expression systems. In such cases, it is necessary to demonstrate the functional equivalence (in terms of physiochemical properties and biological activities) of the proteins purified from the two sources[12].

Refer to the Codex Guideline paragraph 33, for each introduced trait, the expected expression pattern and stability of inheritance is usually demonstrated using data from field trials collected over several seasons and geographical locations. The genomic stability of the insert is usually shown by Southern blotting of DNA extracted from plant material sampled over several seasons and locations. Similarly, stable expression of the inserted DNA is shown by quantification of the corresponding protein or protein activity.

CODEX GUIDELINE PARAGRAPH 33. In addition, information should be provided:

A) to demonstrate whether the arrangement of the genetic material used for insertion has been conserved or whether significant rearrangements have occurred upon integration;
B) to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;
C) to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable through several generations consistent with laws of inheritance. It may be necessary to examine the inheritance of the DNA insert itself or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly;
D) to demonstrate whether the newly expressed trait(s) are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene;
E) to indicate whether there is any evidence to suggest that one or several genes in the host plant has been affected by the transformation process; and
F) to confirm the identity and expression pattern of any new fusion proteins.

[11] For example the FlavrSavr™ tomato, which contains an antisense sequence corresponding to the polygalacturonase encoding gene.

[12] When equivalence is demonstrated based on serological cross-reactivity between the plant and bacterial proteins, it is important to use antisera (either polyclonal or monoclonal antibodies) that have been well characterized with respect to their specificity.

The application of modern profiling technology, such as DNA/RNA microarrays, proteomics, gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to nuclear magnetic resonance (HPLC-NMR), has the potential to broaden the data available for the safety assessment. Sensitive profiling methods may provide indications of minor or major changes at the genome level in mRNA expression or protein production, and/or changes at the level of metabolism. These broad, non-targeted approaches, which do not require prior knowledge of hypothesized changes in levels of particular plant constituents to guide the choice of method, could be of particular interest for foods derived from recombinant-DNA plants modified through the insertion of multiple genes, such as plants with nutritional or health-promoting characteristics (see also the chapter on Evaluation of metabolites).

The utility and applicability of these non-targeted techniques for generating data for risk assessment purposes need further exploration, in particular with respect to establishing and validating the relevance to food safety of any observed changes. One of the major challenges in using these techniques is that observed differences may not be easily distinguishable from natural variations (baseline fluctuations in several thousand variables) in biochemical composition due to the properties of different varieties, the stage of plant development and the health status of the plant, and environmental influences and variations in growth conditions. Profiling methods are not yet suitable for routine risk assessment purposes because the observed variation in profiles cannot be routinely linked to specific biosafety considerations. Further description of baseline ranges, cost reduction, and development and validation of methods are needed.

Randomly generated plant transformation events

The transgene is generally integrated into the host chromosome(s) upon successful application of transformation processes such as the *Agrobacterium*-mediated or biolistics (microprojectile bombardment) methods. Some insertions occur in regions of the plant genome that are not involved in any obvious function, in which case the transgene may express the novel protein as expected without causing unintended change in other plant traits.

When the random insertion occurs in a region of the plant genome that is involved in genome regulation, transcription or protein production, the insertion may lead to unintended plant phenotypes. Each of the plants recovered after the transformation process that is carrying the integrated DNA represents a unique gene transfer "event".

Because insertion of the transgene into the host plant genome occurs randomly, a large number of transformed plants are usually produced initially, each containing single or multiple copies of the transgene. Subsequent small-scale cultivation and selection-based screening will remove unintended phenotypes possessing unwanted traits and/or multiple copy insertion "events" and preserve the most suitable phenotypes for further characterization and further rounds of selection-based breeding to obtain elite cultivars.

Transgene detection using event-specific primers

Two DNA primers (each 20–30 bases long) with nucleotide sequences complementary to the DNA inserted into the recombinant-DNA plant are generally employed in a polymerase chain reaction (PCR) to detect the presence of a transgene. If both of the PCR primers are complementary to the transgene sequence, then all plant varieties and species that carry the same transgene will show the PCR amplification product, irrespective of the location of the insertion in the plant genome. However, it is possible to distinguish among the different insertion "events" of the same transgene in the same plant cultivar by designing the primer pair appropriately.

Event specificity is based on using a primer pair of which one primer is complementary to the plant genomic region adjacent to the point of insertion of the transgene, and the other primer is complementary to a region within the transgene. These primers are known as "event-specific" primers. This primer pair will only amplify a specific insertion "event" because the process of DNA insertion into plants is effectively random. Therefore, each insertion of DNA will take place at random in the plant genome and will lead to that insertion having unique flanking regions of plant DNA.

The use of event-specific primers is necessary for identifying a particular transformation event among other events carrying the same gene in the same host variety or other varieties of the same crop species. Hence, access to sequence information for the flanking regions of the integration site of the inserted DNA is necessary so that regulatory authorities can conduct event-specific monitoring of recombinant-DNA plants. Due to the large variety of plant cultivars harbouring the same transgene, monitoring of recombinant-DNA plants is typically done in two steps. Step one, which is PCR-based, determines the presence of frequently used transgene constructs, and if this is positive, a second-step (also PCR-based) is performed, which employs event-specific primers.

For examples of the use of event-specific primers, see the validated methods published online by the European Commission's Joint Research Centre: http://gmo-crl.jrc.it/default.htm

Extent of refinement at the current level of the technology

Unintended changes can result from the random insertion of DNA sequences into the plant genome, which may cause modifications in the expression of existing genes, or activation of silent genes, possibly resulting in elevated levels of native or new toxins in the food. It is emphasized that the occurrence of unintended effects is not specific to the application of recombinant-DNA technology in plants, as it also occurs in classic plant breeding. In breeding practice, backcrossing and selection based on morphology, yield, crop quality, insect/disease resistance, etc. identify lines with unwanted characteristics that are discarded[13]. Similarly, during the development of recombinant-DNA plants, modified lines that do not meet the expected agronomic, safety and quality requirements will be discarded, resulting in the elimination of many unintended effects from the tissue culture or DNA insertion process[14].

A limitation in the current application of recombinant-DNA technology in plants is the inability to direct the insert DNA (transgene) into a specific genomic location. Further developments in the technology leading to the option to specifically target the DNA insertion to particular genomic regions may eliminate unintended effects such as positional effects on transgene expression and the influence of the insert on plant genome expression ●

[13] Reports of unintended effects that may affect human health are rare, and include examples such as low yields in barley or maize, high content of furanocoumarins in celery, and high glycoalkaloid content in potatoes.

[14] Examples of unintended effects that have been observed in recombinant-DNA plants are potatoes with abnormal tuber tissue or with reduced glycoalkaloid content, soybeans with higher lignin content, and rice with increased Vitamin B6 content or higher levels of certain carotenoid derivatives.

6 Assessment of possible toxicity of foods derived from recombinant-DNA plants

Introduction

Risk assessment also takes into consideration the estimation and assessment of the level and frequency of intake of food from recombinant-DNA plants. This takes into account how frequently and to what extent the population would be exposed to newly expressed substances such as proteins, metabolites or endogenous compounds that are at altered levels in food due to the newly inserted gene (and/or other unintended effects resulting from genetic modification).

Conventional toxicological tests adopted from those originally developed for chemicals (i.e. food additives, pesticides and food contaminants) may be an appropriate approach to determining the safety of newly expressed substances. It is possible to determine the NOEL (no observed [adverse] effect level) of the new substance and subsequently the safety factor related to the level of exposure expected in the general population. Hence the safety factor is applied to derive the acceptable or tolerable daily intake. If such studies are to be undertaken, they should be designed according to the identity and biological function of the substances under consideration.

Conventional toxicology studies on the safety of whole foods are, however, not meaningful in practice because foods are complex mixtures of compounds characterized by wide variation in composition and nutritional value. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. These difficulties in applying conventional toxicology approaches to recombinant-DNA plants have led to the development of the concept of substantial equivalence. This conceptual approach acknowledges that the goal of the assessment is not to establish absolute safety but to consider whether foods derived from recombinant-DNA plants are as safe as their traditional counterparts or not.

Conceptual approach to toxicity studies

The conceptual approach to the assessment of potential toxic properties of food involves biochemical characterization of the novel product from the inserted DNA element by *in vitro* digestibility studies, determination of the amino acid sequence similarity to known toxins, and acute oral toxicity studies based on an animal model. If on the basis of these studies a longer-term effect can be assumed then additional subchronic and chronic toxicity testing will be required. The *in vitro* digestibility studies are performed to determine the resistance of the novel product to acid, thus simulating the conditions in gastric and intestinal fluids. The sequence of the six amino terminal amino acids is compared with the amino terminal of the amino acid sequence of known toxins to determine their similarity. If the similarity is significant, it is possible that the novel product from the inserted gene is a toxin. The novel product is then subjected to subchronic toxicological studies to determine the safety factor for consumption relative to the exposure of the general population.

CODEX GUIDELINE PARAGRAPH 34. *In vitro* nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in plants. The new substances can be conventional components of plant foods such as proteins, fats, carbohydrates or vitamins which are novel in the context of that recombinant-DNA plant. New substances might also include new metabolites resulting from the activity of enzymes generated by the expression of the introduced DNA.

CODEX GUIDELINE PARAGRAPH 35. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations and mean values. Current dietary exposure and possible effects on population sub-groups should also be considered.

CODEX GUIDELINE PARAGRAPH 36. Information should be provided to ensure that genes coding for known toxins or anti-nutrients present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic or anti-nutritious characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, since conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate anti-nutrients or toxicants.

CODEX GUIDELINE PARAGRAPH 37. For the reasons described in Section 3, conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary.

CODEX GUIDELINE PARAGRAPH 38. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (e.g. protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[15] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, and taking into account its biological function in the plant where known.

CODEX GUIDELINE PARAGRAPH 39. Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the plant of the substance and dietary exposure. The type of studies to be performed may include studies on metabolism, toxicokinetics, sub-chronic toxicity, chronic toxicity/carcinogenicity, reproduction and development toxicity according to the traditional toxicological approach.

CODEX GUIDELINE PARAGRAPH 40. This may require the isolation of the new substance from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be biochemically, structurally, and functionally equivalent to that produced in the recombinant-DNA plant.

[15] Guidelines for oral toxicity studies have been developed in international fora, for example, the OECD Guidelines for the Testing of Chemicals.

The conceptual approach to evaluating the toxicity of an introduced substance is described in Codex Guideline paragraphs 34–40.

Methods used to determine absence of toxicity

The requirements for and methods used to determine whether the new substance from the inserted gene is a toxin or not are described in the Codex Guideline paragraphs 34–4. Large amounts of purified protein expressed by the transgene are required for toxicity studies. The levels obtainable in plant tissue are generally not sufficient, and the proteins are therefore usually extracted from GM micro-organisms (such as *Escherichia coli*) engineered to express the protein in large amounts. In such cases, biochemical and functional equivalence of the bacterially derived version and the plant-expressed version must be demonstrated.

Animal feeding studies are usually performed to establish the absence of acute and subchronic toxicity. Animal feeding studies nevertheless have recognized limitations. It is important to realize that whereas carefully performed animal feeding studies demonstrating a lack of effect on selected physiological outcomes can be useful, the studies do not provide complete assurance of safety, because of the usual caveats with extrapolating results from other animals to humans. The results should be considered as “confirmatory” and “safety assuring”

CODEX GUIDELINE PARAGRAPH 10. The use of animal models for assessing toxicological endpoints is a major element in the risk assessment of many compounds such as pesticides. In most cases, however, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. It is therefore relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

CODEX GUIDELINE PARAGRAPH 11. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, often characterized by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects which are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

CODEX GUIDELINE PARAGRAPH 12. Due to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods derived from food plants, including recombinant-DNA plants. This has been addressed by the development of a multidisciplinary approach for assessing safety which takes into account both intended and unintended changes that may occur in the plant or in the foods derived from it, using the concept of substantial equivalence.

and are an additional component of the overall safety assessment in those circumstances in which they are warranted. The advantages and limitations of animal studies that must be taken into consideration in the determination of the safety of the foods derived from recombinant-DNA plants are discussed in the Codex Guideline paragraphs 10–12.

Feeding studies that use whole foods rather than isolated compounds may be appropriate when there are significant compositional changes in the food derived from recombinant-DNA plants; see Codex Guideline paragraph 53.

The ethical aspects of and necessity for animal feeding studies are issues that must be continually reconsidered to avoid unnecessary animal suffering. The Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology in 2000 (*Safety aspects of genetically modified foods of plant origin,* Section 4.2, paragraph 4.2.2) provided a useful discussion of the need for animal studies (Box 6.1).

It is generally considered that a subchronic study in rodents of 90 days' duration is the minimum requirement to demonstrate the safety of repeated consumption of foods derived from recombinant-DNA plants in the diet. The Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology in 2000 (*Safety testing of food additives and contaminants and the long-term evaluation of foods produced by biotechnology*, page 4) provided a useful discussion of subchronic toxicity studies (summarized in Box 6.2).

The document produced by the United States Food and Drug Administration on the toxicological principles of the safety assessment of food ingredients (US FDA, 2003)

CODEX GUIDELINE PARAGRAPH 53. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.

Box 6.1. Need for animal studies (FAO/WHO, 2000)

If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, animal testing may be deemed necessary. This would particularly be the case if the food were expected to make a significant dietary contribution, if there is no history of consumption of the novel gene product or if the modification affects several metabolic pathways.

In the situation where the genetically modified food differs from the traditional counterpart by the presence of one or a few new genes and their products, it may be possible to isolate and study these in a manner analogous to conventional toxicity testing of food additives.

However it is essential to ensure that the material tested is biochemically and functionally equivalent to that produced in the genetically modified food. This provides the possibility of increasing the sensitivity of toxicity tests compared with that possible if the products of the genetically modified plants had been fed directly and avoids some of the artefacts that can occur in toxicity tests on whole foods. However, this strategy is only applicable if the preceding detailed analysis does not reveal significant changes other than those expected. Otherwise testing of the whole food may be required. When animal testing is conducted on the whole food, it should generally be on the food as consumed by humans. The type of animal study would need to be considered on a case by case basis. In addition to investigating potential toxicological effects, animal studies may also be required if the genetic modification directly or indirectly affects the content or bioavailability of nutrients.

Where toxicological studies are considered necessary to assess the safety of long term consumption of a food in the diet, it is generally considered that a sub-chronic study of 90-days duration is the minimum requirement to demonstrate the safety of repeated consumption of a food in the diet. This may need to be preceded by a pilot study of short duration to ensure that the diet is palatable to the test species and that the levels of incorporation of the test article are appropriate, e.g. the control diet containing the equivalent level of the comparator does not produce effects, as a result of normal levels of natural toxicants present in traditional foods accepted as safe. The highest dose level used in any animal study should be the maximum achievable without causing nutritional imbalance while the lowest level used should be comparable to the anticipated human intake.

The need for additional toxicological tests should be considered on a case-by-case basis taking into account the results of the 90-day study and other studies. For example, proliferative changes in tissues during the 90-day study may indicate the need for a longer-term toxicity study.

Conventional toxicological tests are of limited value in assessing whole foods, including genetically modified foods. Based on the maximum levels of the whole food that can be incorporated into experimental diets as indicated previously, a margin of safety may be estimated based on the absence or nature of adverse effects and likely human exposure. Improved experimental designs should take into account the need for nutritionally adequate animal diets, avoiding some of the inappropriate testing of foods or products.

It has been suggested that the use of biomarkers of early effects might increase diagnostic value and sensitivity of toxicity tests on foods (Schilter *et al.*, 1996). However, it will be necessary not to confuse adaptive and toxic effects in applying this approach.

Box 6.2. Toxicological studies on foods produced by biotechnology (FAO/WHO, 2000)

When a food product of biotechnology differs from a traditional food in a few well defined characteristics, these may serve to focus the safety evaluation process and determine the tests required. The toxicological focus will be on the few well defined characteristics. It may be possible to isolate and study differences in one or a few new genes and their products in a manner analogous to conventional toxicity testing of food additives.
The conventional toxicity testing of these new genes and their products is usually the standard 14-day subacute study (OECD, 1995: Guideline 407).
A substance to be tested for toxicity is usually fed to rats in a standard 14-day subacute study at a level that would reflect a very large margin of safety. The NOEL would represent the maximum level that can be incorporated into experimental diets with no adverse effects, and this could be translated to the safety factor for human exposure to the product.
Human studies should contribute to the evaluation process, and might be conducted when the i*n vivo* animal studies demonstrate no unexpected or irreversible effects[16].

A tiered approach to such studies should be adopted to investigate tolerance up to maximum levels of potential intake. The purpose is to have some confirmatory controlled clinical studies before getting into the greater complexities of general release. It is desirable that human studies are conducted as soon as possible within ethical constraints in order better to target animal studies and to avoid extensive but irrelevant animal studies. Observations from animal and human studies may reveal that the food is safe for its intended use, or may reveal unexpected indications that require more detailed investigation to confirm food safety.

[16] Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, Topic 6: Safety testing of food additives and contaminants and the long term evaluation of foods produced by biotechnology. 29 May–2 June 2000.

Box 6.3. Technical aspects of subchronic toxicity studies (FDA, 2003)*

Subchronic toxicity studies with rodents are generally conducted for between 90 days (3 months) and 12 months. Subchronic toxicity studies are generally used to help predict appropriate doses of the test substance for future chronic toxicity studies, to determine NOELs for some toxicology endpoints or to allow future long-term toxicity studies in rodents and non-rodents to be designed with special emphasis on identified target organs. They cannot be used to determine the carcinogenic potential of a test substance.

It is essential that all non-clinical laboratory studies are conducted according to the internationally recognized guidelines[17] and good laboratory practice (GLP)[18] regulations. Other factors that must be taken into consideration are discussed below.

Test animals

The care, maintenance and housing of laboratory animals must follow the guidelines in the *Guide for the care and use of laboratory animals*[19].

The selection of species, strains and sex must take into consideration of test animals' general sensitivity. The responsiveness of particular organs and tissues of the test animals to the toxic substance to be tested must be considered when selecting rodent species, strains and substrains for toxicity studies. The selection of inbred, outbred or hybrid rodent strains for toxicity studies should be based upon the scientific questions to be answered. Moreover, the test animals should come from well characterized and healthy colonies, because recent information has suggested problems with the survivability of some strains of rats and test animals should be selected to achieve the recommended duration of the study.

The age of the test animals may result in variation in results. Testing should be conducted on young animals, and dosing should be commenced immediately after weaning, following an acclimation period of at least 5 days, and for rodents no later than 6–8 weeks of age.

An equal number of males and females of each species and strain should be used for the study. For subchronic toxicity studies, experimental and control groups should contain at least 20 rodents of each sex per group. These recommendations will help ensure that the number of animals that survive until the end of the study will be sufficient to permit a meaningful evaluation of toxicological effects.

The animals should be housed one per cage in order to address the following concerns.

If more than one animal is present in a cage, the feed efficiency (the relationship between feed consumed and body weight gained) cannot be determined with accuracy.

It is impossible to determine whether a decrease in body weight is due to decreased palatability or substance-mediated toxicity.

The organs and tissues from moribund and dead animals may be lost as a result of cannibalism if they are not individually caged.

The diet provided to the animals must be isocaloric and contain the same levels of nutrients (e.g. fibre and micronutrients) in both the treated and the control groups[20]. Inadequately controlled dietary variables may result in nutritional imbalances or caloric deprivation that could confound interpretation of the results of the toxicity study and alter the outcome and reproducibility of the studies.

The animals should be assigned to control and compound-treated groups in a stratified random manner; this will minimize bias and assure comparability of pertinent variables across treated and control groups (for example, mean body weight and body weight ranges). If other characteristics are to be used as the basis for randomization then that characterization should be described and justified. Animals in all groups should be placed in the study on the same day; if this is not possible because of the large number of animals in a study, animals may be placed in the study over several days. If recruitment to the study over several days is selected, a preselected portion of the control and experimental animals should be placed in the study on each day in order to maintain concurrence.

Experimental design

The animals should be exposed to the test substance on 7 days per week for a minimum of 90 consecutive days (3 months).

The route of administration of the test substance should be appropriate to the normal human exposure. A justification must be provided if alternative routes are used. Possible administration routes are described below.

The substance should be administered in the diet if the human exposure is likely to be through consumption of solid foods or a combination of solid and liquid foods. The animals should not be allowed to consume selectively either the basal diet or the test substance in the diet. Care must be taken to ensure that processes used during pelleting, such as heating, do not affect the test substance.

The test substance may be administered by dissolving in the drinking water. Alternatively, the test substance may be administered by encapsulation or oral intubation (gavage) if the human exposure is expected to be through daily ingestion of a single large dose instead of continual ingestion of small doses. Administration by gavage should be performed at approximately the same time each day, and the maximum volume of solution to be given by gavage in one dose should depend on the size of the test animal. In rodents, the volume should not exceed 1 ml/100 g body weight and for oily substances it should not exceed 0.4 ml/100g body weight. If the administered amount is to be divided into smaller doses, all must be administered within a 6-hour period.

Dose groups

At least three dose levels of the test substance should be used per sex (one dose level per group); however, ideally, four or five dose levels of the test substance should be used. A concurrent control group should be included. The appropriate dose levels for subchronic toxicity studies can be determined based on the information from acute and short-term toxicity studies.

(Continued)

17 OECD Guideline for the testing of chemicals, repeated dose 90-day oral toxicity study in rodents, 407, Sept. 1998.

18 OECD Principles of Good Laboratory Practice Directive 87/18/EEC, Directive 88/320/EEC.

19 National Research Council Institute of Laboratory Animal Resources. 1996. Guide for the care and use of laboratory animals. Washington, DC, National Academy Press.

20 Nutrient requirements of laboratory animals, 4th Revised Edition, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council, 1995.

Box 6.3 (cont.)

Selection of treatment doses
A minimum of three dose levels of the test substance and a concurrent control group should be used in toxicity studies. The three dose levels administered should follow the guidelines as follows:

- the high dose should be sufficiently high to induce a toxic response in the test animals;
- the intermediate dose should be sufficiently high to elicit minimal toxic effects in the test animals, such as alterations in enzyme levels or a slight decrease in body weight gain;
- the low dose should not induce toxic responses in the test animals.

Controls
A concurrent control group of test animals is required. The control group in dietary studies should be fed the basal diet.

The carrier or vehicle for the test substance should be given to control animals at a volume equal to the maximum volume of carrier or vehicle given to any dosed group of animals. Information on the toxicity of the carrier or vehicle should be available to ensure that it will not compromise the results of the study.

Observations and clinical tests: observations of test animals
Observations should be made of all animals at least once or twice a day throughout the study for general signs of pharmacological and toxicological effects, morbidity and mortality. The usual interval between observations should be at least 6 hours. Individual records should be maintained for each animal and the time of onset and characteristics and progression of any effects should be recorded, preferably using a scoring system. The clinical evaluations should not only assess the general pharmacological and toxicological effects but also neurological disorders, behavioural changes, autonomic dysfunction, and other signs of nervous system toxicity. The signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and other evidence of autonomic activity. In addition, changes in posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes or bizarre behaviour should be recorded. The development of tumours should be recorded, particularly in long-term studies. During the course of a study, toxic and pharmacological signs may suggest the need for additional clinical tests or expanded post-mortem examinations.

Body weight and feed intake data
Test animals should be weighed at least once a week. Feed consumption (or water consumption if the test substance is administered in the drinking water) should be measured every week during a subchronic toxicity study.

Clinical testing
The following tests should be performed: ophthalmological examination, haematology profiles, clinical chemistry tests, urinalyses, neurotoxicity screening/testing and immunotoxicity studies.

Necropy and microscopic examination
All test animals should be subjected to the following examinations: gross necropsy, measurement of organ weight, preparation of tissues for microscopic examination, microscopic evaluation, and histopathology of lymphoid organs.

***Reference:* US FDA.** 2003. *Toxicological principles for the safety assessment of food ingredients: Red Book 2000, November 2003. IV.C.4a. Subchronic toxicity studies with rodents.* Food and Drug Administration, Center for Food Safety and Applied Nutrition, Department of Health and Human Services.

may also be a useful source for the technical aspects of subchronic toxicity studies (summarized in Box 6.3).

Chronic toxicity studies

Chronic toxicity studies involve long-term administration of the test substance, usually in the diet or drinking water, and sometimes by gavage. Chronic toxicity studies are designed to detect possible cumulative effects on target organ(s) in a dose–response dependent manner. The need for long-term chronic toxicity studies should be addressed on a case-by-case basis, and only when the results of the 90-day or other feeding studies indicate the need to consider toxicity from a longer term-perspective.

Quality assurance

It is very important that the organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported are conducted according to the principles of good laboratory practice (GLP)[18]. The principles of GLP must be applied to

[18] See page 28.

testing of chemicals to generate data on their properties and/or their safety for human health or the environment. In toxicology studies, it is essential to be certain that the data used to estimate safety are of a quality that is acceptable to all parties. It is also important in toxicology studies to establish the relationship between the changes in physiological parameters measured and the dose levels of the tested compound to which animals are exposed. Hence, good quality data are of the utmost importance and lead to accurate interpretation of the toxicity and estimation of the NOEL of the tested compound. From this interpretation, the safety factor can be established by estimating the maximum levels to which the human population can be exposed without observed adverse effects on health. Moreover, any observed differences between treated and untreated animals in the physiological parameters measured in animal experiments must be analysed statistically to establish the confidence limits of these differences.

References

Doerfler, W. 2000. *Foreign DANN in mammalian systems*. Wennheim, Germany, Wiley-VCH. 181 pp.

FAO/WHO. 2000. *Safety aspects of genetically modified foods of plant origin*. Joint FAO/WHO Expert Consultation on foods derived from biotechnology, 29 May–2 June 2000, Geneva, Switzerland. ftp://ftp.fao.org/docrep/nonfao/ae584e/ae584e00.pdf

FAO/WHO. 2000. *Safety testing of food additives and contaminants and the long-term evaluation of foods produced by biotechnology*. Topic 6. Joint FAO/WHO Expert Consultation on foods derived from biotechnology, 29 May–2 June 2000, Geneva, Switzerland. ftp://ftp.fao.org/es/esn/food/Bio-08.pdf

OECD. 1995. *Guideline for the testing of chemicals, Guideline 407. Repeated dose 28-day oral toxicity study in rodents*. Paris, Organization for Economic Co-operation and Development. http://www.oecd.org/dataoecd/50/18/37478478.pdf

OECD. 1998. *OECD series on principles of good laboratory practice and compliance monitoring number 1*. ENV/MC/CHEM(98)17. Paris, Organization for Economic Co-operation and Development. http://www.olis.oecd.org/olis/1998doc.nsf/LinkTo/env-mc-chem(98)17

OECD. 2000. *Report of the task force for the safety of novel foods and feeds*. C(2000)86/ADD1. Paris, Organization for Economic Co-operation and Development. http://www.olis.oecd.org/olis/2000doc.nsf/LinkTo/C(2000)86-ADD1

Schilter, B., Holzhäuser, D., Cavin, C. & Huggett, A.C. 1996. An integrated in vivo and in vitro strategy to improve food safety evaluation. *Trends Food Sci. Technol.*, 7: 327–332.

US FDA. 2003. *Toxicological principles for the safety assessment of food ingredients: Red book 2000, November 2003. IV.C.4a. Subchronic toxicity studies with rodents*. Washington DC, USA, United States Food and Drug Administration, Center for Food Safety and Applied Nutrition, Department of Health and Human Services.

US National Research Council. 1995. *Nutrient requirements of laboratory animals*, 4th Revised Edition. Washington DC, USA, Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board of Agriculture ●

7 Assessment of possible allergenicity (Proteins) in foods derived from recombinant-DNA plants

Food allergies

Food allergies are adverse reactions to an otherwise harmless food or food component and involve an abnormal response of the body's immune system to specific protein(s) in foods known as "allergens". True food allergies may involve several types of immunological response (Sampson and Burks, 1996).

The most common types of food allergies are mediated by allergen-specific immunoglobulin E (IgE) antibodies[21]. IgE-mediated reactions are known as immediate hypersensitivity reactions because symptoms occur within minutes to a few hours after ingestion of the offending food. IgE-mediated reactions can occur to pollens, mould spores, animal danders, insect venoms and other environmental stimuli as well as foods. IgE-mediated reactions affect perhaps 10 to 25 percent of the population in developed countries (Mekori, 1996).

Food allergies represent a small fraction of all allergic diseases, affecting less than 2.5 percent of the population in developed countries (Anderson, 1996). Infants and young children are more commonly affected by IgE-mediated food allergies than adults; the prevalence among infants under the age of 3 years may be as high as 5 to 8 percent (Bock, 1987; Sampson, 1990).

True food allergies also include cell-mediated reactions, which involve sensitized tissue-bound lymphocytes rather than antibodies (Sampson, 1990). In cell-mediated reactions, the onset of symptoms occurs more than 8 hours after ingestion of the offending food. The role of foods in cell-mediated reactions remains uncertain (Burks and Sampson, 1993) but coeliac disease[22], also known as gluten-sensitive enteropathy, affects one in every 300 to 3 000 individuals in the population, depending upon the specific geographical region. Both IgE-mediated food allergies and gluten-sensitive enteropathy are treated with specific avoidance diets. Because in both cases the threshold dose is quite low, great care must be taken in the construction of safe and effective avoidance diets.

The Codex Alimentarius Commission has produced a list of the most common allergenic foods associated with IgE-mediated reactions on a worldwide basis, which includes peanuts, soybeans, milk, eggs, fish, crustacea, wheat and tree nuts. These commonly allergenic foods account for over 90 percent of all moderate to severe allergic reactions to foods, although an extensive literature search has revealed more than 160 foods associated with sporadic allergic reactions (Hefle *et al.*, 1996).

Allergic reactions to fresh fruits and vegetables, comprising the so-called oral allergy syndrome, are also rather common (Parker *et al.*, 1990), but these foods are not included on the Codex Alimentarius Commission list because the symptoms are typically mild and confined to the oropharyngeal region, and the allergens are unstable to heating and digestion. The list established by the Codex Alimentarius Commission also includes gluten-containing cereals (wheat, rye, barley, oats and spelt) that are implicated in the aetiology of gluten-sensitive enteropathy. Table 7.1 provides a summary of protein sequences of food allergens from foods of plant origin and their accession numbers for retrieving the sequence data from the relevant databases.

[21] IgE, or immunoglobulin E, is a protein antibody that recognizes an allergen. IgE circulates in the blood and becomes fixed on the surface of specific cells (basophils and mast cells). When IgE on the cell surface binds to an allergen, this triggers the release of chemical mediators that provoke the symptoms associated with allergic reactions.

[22] Gluten-sensitive enteropathy is a malabsorption syndrome characterized by body wasting, anaemia, diarrhoea and bone pain, along with other symptoms.

Almost all food allergens are proteins, although it is possible that other food components may act as haptens[23]. Similarly, prolamin proteins from wheat, rye, barley, etc. are involved in the elicitation of gluten-sensitive enteropathy. While the crops from which staple foods are derived contain tens of thousands of different proteins, relatively few are allergenic. The distribution of these proteins varies throughout the plant and can be influenced by environmental factors, such as climate and disease stress. Conventional breeding removes diversity from or introduces protein diversity into the food supply, but has had little, if any, effect on the allergenic potential of our major foods.

Table 7.1. Food allergen protein sequences of plant origin[1]

Species	*Common name*	*Allergen*	*Synonym/function*	*Accession[2]*
Arachis hypogea	Peanuts	*Ara* h 1	Clone P41b	L34402
			Clone 5A1	L33402
			Clone P17	L38853
		Peanut lectin	Agglutinin	S14765
Bertholletia exceisa	Brazil nut	*Ber* e 1	2S albumin (BE2S1 gene)	X54490
Brassica juncea	Leaf mustard	*Bra* j IE-L	2S albumin large chain	S35592
		Bra j IE-S	2S albumin small chain	S35591
Carica papaya	Papaya	Papain		M15203
Glycine max	Soybean	Glycinin	A1aBx subunit	X02985
			A2B1a subunit	Y00398
			A3B4 subunit	M10962
			G1 subunit	X15121
			G2 subunit	X15122
			G3 subunit	X15123
		beta-Conglycinin	alpha-subunit	X17698
			CG4 subunit	S44893
		Soy lectin	Soy agglutinin	K00821
		Kuntz trypsin inhibitor	KTi-s subtype	X80039
			KTi-a subtype	X64447
			KTi-b subtype	X64448
Hordeum vulgare	Barley	*Hor* v 1	alpha-amylase/trypsin inhibitor	S26197
		Hor v 1	alpha-amylase/trypsin inhibitor	P32360
Malus domestica	Apple	*Mal* d 1	Profilin	X83672
Oryza sativa	Rice	RAP	Rice allergenic protein	X66257
		RAG1	Rice allergen 1	D11433
		RAG2	Rice allergen 2	D11434
		RAG5	Rice allergen 3	D11430
		RAG14	Rice allergen 14	D11432
		RAG17	Rice allergen 17	D11431
Phaseolus vulgaris	Kidney bean	PR-1	Pathogenesis related protein 1	S11929
		PR-2	Pathogenesis related protein 2	S11930
Sinapis alba	White mustard	*Sin* a 1.1	2S albumin/amylase inhibitor	S54101
		Sin a 1.2	2S albumin/amylase inhibitor	PC1247
Triticum aestivum		WGA	Wheat germ agglutinin A	M25536
		WGA	Wheat germ agglutinin D	M25537
Triticum durum	Pasta wheat	WGA	Wheat germ agglutinin	J02961
Triticum turgidum	Poulard wheat	16K allergen	alpha-amylase inhibitor	S19296

[1] *Adapted from Metcalfe* et al. *(1996).*

[2] *Public domain databases: GenBank/EM BL/Genpept ver 86.0, SWISSPROT ver 30, PIR ver 41.*

[23] Haptens are small molecules that may interact with body proteins or food proteins and cause these proteins to become allergenic.

Allergenicity potential of foods derived from recombinant-DNA plants

Potential allergenicity is a concern with proteins introduced into the human diet through food derived from recombinant-DNA plants, especially when there is no history of their consumption, where the source cannot be readily identified, or when they are recombined versions of proteins from different sources. The current allergenicity assessment approach is presented in the Annex "Assessment of possible allergenicity" of the Codex Guideline (see Appendix 2). As there is no definitive test that can be relied upon to predict allergic responses in humans to a newly expressed protein, the Codex recommends that an integrated, stepwise, case-by-case approach be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data because no single criterion is sufficiently predictive.

In addition to the Annex, the Codex Guideline outlines approaches to allergenicity assessment in paragraphs 41–43.

Allergenicity assessment strategy

The initial steps in assessing the possible allergenicity of any newly expressed protein are the determination of the source of the introduced protein, any significant similarity between the amino acid sequence of the protein and that of known allergens, and its structural properties, including, but not limited to, its susceptibility to enzymatic degradation, heat and/or acid and enzymatic treatment.

As there is no single test that can predict the likely human IgE response to oral exposure, the first step in the characterization of newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens using a weight of evidence approach (see Box 7.1 for an outline of some important parameters used). This will require the isolation of any newly expressed proteins from the recombinant-DNA plant or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA plant. Particular attention should be paid to the choice of the expression host, because the post-translational modifications allowed by different hosts (i.e. eukaryotic vs prokaryotic systems) may have an impact on the allergenic potential of the protein.

It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

CODEX GUIDELINE PARAGRAPH 41. When the protein(s) resulting from the inserted gene is present in the food, it should be assessed for potential allergenicity in all cases. An integrated, stepwise, case-by-case approach used in the assessment of the potential allergenicity of the newly-expressed protein(s) should rely upon various criteria used in combination (since no single criterion is sufficiently predictive on either allergenicity or non-allergenicity). As noted in paragraph 20, the data should be obtained using sound scientific methods. A detailed presentation of issues to be considered can be found in the Annex to this document.

CODEX GUIDELINE PARAGRAPH 42. The newly expressed proteins in foods derived from recombinant-DNA plants should be evaluated for any possible role in the elicitation of gluten-sensitive enteropathy, if the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains.

CODEX GUIDELINE PARAGRAPH 43. The transfer of genes from commonly allergenic foods and from foods known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

Box 7.1. Important parameters used in the assessment of allergenicity

Source of the protein

As part of the database supporting the safety of foods derived from recombinant-DNA plants, any reports of allergenicity associated with the donor organism should be described. Allergenic sources of genes are defined as those organisms for which reasonable evidence of IgE-mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include the availability of sera for screening purposes, documentation of the type, severity and frequency of allergic reactions, the structural characteristics and amino acid sequence of the protein, and the physiochemical and immunological properties (if available) of known allergenic proteins from that source.

Amino acid sequence homology

The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether the protein has allergenic potential. Sequence homology searches should be performed to compare the structure of all newly expressed proteins with all known allergens. Searches should be conducted using various algorithms such as FASTA or BLASTP[24] to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed to identify sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results[25]. Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35 percent identity in a segment of 80 or more amino acids (FAO/WHO, 2001), or when other scientifically justified criteria are met. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding specifically with IgE antibodies.

A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive with known allergens. A result indicating the absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also Specific serum screening, below). A finding of positive sequence homology indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

Pepsin resistance

Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential[26]. Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude the possibility that the newly expressed protein could be a relevant allergen. Although the pepsin resistance protocol is recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided[27].

Specific serum screening

For those proteins that originate from a source known to be allergenic, or that have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals[28]. In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available, as described in the final paragraph below.

In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols[29]. A positive result in such tests would indicate a potential allergen.

[24] FASTA is a computer program, based on the method of W. Pearson and D. Lipman (*Proc. Natl. Acad. Sci. USA*, 85: 2444–2448, 1988), that searches for similarities between one sequence (the query) and any group of sequences (the database) (http://fasta.bioch.virginia.edu/). The BLAST (basic local alignment search tool) program uses a strategy based on matching sequence fragments by employing a powerful statistical model, developed by S. Karlin and S. Altschul (*Proc. Natl. Acad. Sci. USA*, 87: 2264–2268, 1990), to find the best local alignments. BLASTP is the NCBI BLAST program for comparing a protein query sequence to a protein database. The original BLAST program was developed at NCBI (http://www.ncbi.nih.gov/BLAST/). There is a separate BLAST distribution called WU-BLAST available from Washington University (http://blast.wustl.edu/).

[25] It is recognized that the 2001 FAO/WHO consultation suggested moving from eight to six identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives; conversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison (FAO/WHO, 2001).

[26] The method outlined in the United States Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood *et al.* 1996).

[27] Report of the Joint FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology (FAO/WHO, 2001): Section 6.4 Pepsin resistance.

[28] According to the Joint Report of the FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology (FAO/WHO, 2001) a minimum of eight relevant sera is required to achieve 99 percent certainty that the new protein is not an allergen, in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

[29] An *ex vivo* procedure is described as testing for allergenicity performed using cells or tissue culture from allergic human subjects (FAO/WHO, 2001).

The level of exposure to the newly expressed protein and the effects of relevant food processing will contribute towards an overall conclusion about the potential human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing that would be applied and its effects on the presence of the protein in the final food product.

As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include targeted serum screening (i.e. the assessment of protein binding to IgE in sera of individuals with clinically validated allergic responses to broadly related categories of foods), the development of international serum banks, use of animal models, and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

References

Anderson, J.A. 1996. Allergic reactions to foods. *Critical Rev. Food Sci. Nutr.*, 36: S19–S38.

Astwood, J.D., Leach, J.N. & Fuchs, R.L. 1996. Stability of food allergens to digestion *in vitro*. *Nature Biotech.*, 14: 1269–1273.

Bock, S.A. 1987. Prospective appraisal of complaints of adverse reactions to foods in children during the first three years of life. *Paediatrics* 79: 683–688.

Burks, A.W. & Sampson, H. 1993. Food allergies in children. *Curr. Prob. Paediatrics* 23: 230–252.

FAO/WHO. 2001. *Evaluation of allergenicity of genetically modified foods*. Report of a joint FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology. 22–25 January 2001. Rome, Italy, Food and Agriculture Organization of the United Nations.

Hefle, S.L., Nordlee, J.A. & Taylor, S.L. 1996. Allergenic foods. *Critical Rev. Food Sci. Nutr.*, 36: S69–S89.

Mekori, Y.A. 1996. Introduction to allergic disease. *Critical Rev. Food Sci. Nutr.*, 36: S1–S18.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. & Fuchs, R.L. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Rev. Food Sci. Nutr.*, 36: S165–S186.

Parker, S.L., Leznoff, A., Sussman, G.L., Tarlo, S.M. & Krondl, M. 1990. Characteristics of patients with food-related complaints. *J. Allergy Clin. Immunol.*, 86: 503–511.

Sampson, H.A. 1990. Immunologic mechanisms in adverse reactions to foods. *Immunol. Allergy Clin. N. Am*. 11: 701–706.

Sampson, H.A. & Burks, A.W. 1996. Mechanisms of food allergy. *Ann. Rev. Nutr*. 16: 161–177.

Additional resources

International Food Biotechnology Council and International Life Sciences Institute Allergy and Immunology Institute. 1996. Allergenicity of foods produced by genetic modification. F.M. Clydesdale, ed. *Crit. Rev. Food Sci. Nutr.*, 36.

OECD. 1997. *Safety assessment of new foods: results of an OECD survey of serum banks for allergenicity testing, and use of databases.* Paris, Organization for Economic Cooperation and Development (OECD). http://www.olis.oecd.org/olis/1997doc.nsf/LinkTo/NT00000C6A/$FILE/JT00121603.PDF

Taylor, S. 2002. *Topic 13: Allergenicity*. Joint FAO/WHO Expert Consultation on foods derived from biotechnology. Geneva, WHO/FAO ●

8. Compositional analyses of key components, evaluation of metabolites, food processing and nutritional modification

Compositional analysis

Food composition analysis is concerned with both beneficial and harmful components in the human diet: nutrients, bioactive non-nutrients, antinutrients, toxicants, contaminants and other potentially useful and dangerous elements. The composition of any food varies, and the differences are caused by plant variety, growth and storage conditions, climate, processing and several other factors. As a result, compositional data are used mainly as an estimate or starting point to guide further analysis, if deviations from expectations are seen.

Possible changes in the composition of the recombinant-DNA plant are assessed using comparative analyses of the key nutrients, antinutrients, toxicants and other important components of the crop with the corresponding compounds in an appropriate comparator crop. Data on the composition of recombinant-DNA plants and their conventional counterparts are obtained from samples produced in controlled field trials and analysed using validated methods and appropriate statistical techniques. Samples are normally analysed in a random order using the same methods in order to prevent bias.

Based on the comparative approach, it is important to decide which nutrients the evaluation should be focused on. Generally, the food safety assessment considers the potential for any change in the concentration of key elements that have a significant impact on the diet, as well as the potential for any change in the bioavailability of key nutritional components.

Key compositional data that are statistically non-distinguishable collected from both the recombinant-DNA crop plant and the isogenic counterpart, grown under near identical conditions, are essential to establish substantial equivalence. Moreover, the compositional data should be shown to fall within the published range for conventional varieties that are considered to be safe for consumption based on a history of safe use.

Where significant changes are detected, analytical methods traditionally applied in the evaluation of food constituents, such as measurement of total protein, fat, ash, fibre and micronutrients, may need to be augmented with additional analyses to identify the nature of the changes observed, and to determine whether the observed differences could adversely affect health. Paragraphs 44 to 46 of the Codex Guideline outline the key considerations for key components and metabolites in recombinant-DNA plants.

There may be instances where reference values are not available for a particular food crop e.g. crops that are nutritionally modified and/or indigenous to a specific region. In such cases, the purpose of the assessment is to gather data to establish a compositional profile. It is important to note that all plant breeding methods, conventional and modern, have the potential to alter the compositional profile and nutritional value of plants or lead to unexpected or unintended changes in concentrations of various natural toxicants or antinutrients[30].

Unintended changes in levels of nutrients can theoretically arise in several ways. Insertion of genetic material could disrupt or alter the expression of normally expressed plant genes. Expression of the introduced gene - through protein synthesis - might lead to enzymatic activity and substrate ranges beyond the intended target molecule, and a high transgene expression

[30] International Food Composition Tables Directory, see "additional resources" section.

CODEX GUIDELINE PARAGRAPH 44. Analyses of concentrations of key components[31] of the recombinant-DNA plant, and especially those typical of the food, should be compared with an equivalent analysis of a conventional counterpart grown and harvested under the same conditions. In some cases, a further comparison with the recombinant-DNA plant grown under its expected agronomic conditions may need to be considered (e.g. application of an herbicide). The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. The comparator(s) used in this assessment should ideally be the near isogenic parental line.
In practice, this may not be feasible at all times, in which case a line as close as possible should be chosen. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that are nutritionally important or that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

CODEX GUIDELINE PARAGRAPH 45. The location of trial sites should be representative of the range of environmental conditions under which the plant varieties would be expected to be grown. The number of trial sites should be sufficient to allow accurate assessment of compositional characteristics over this range. Similarly, trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature. To minimise environmental effects, and to reduce any effect from naturally occurring genotypic variation within a crop variety, each trial site should be replicated. An adequate number of plants should be sampled and the methods of analysis should be sufficiently sensitive and specific to detect variations in key components.

CODEX GUIDELINES PARAGRAPH 46. Some recombinant-DNA plants may have been modified in a manner that could result in new or altered levels of various metabolites in the food. Consideration should be given to the potential for the accumulation of metabolites in the food that would adversely affect human health. Safety assessment of such plants requires investigation of residue and metabolite levels in the food and assessment of any alterations in nutrient profile. Where altered residue or metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g. procedures for assessing the human safety of chemicals in foods).

level might reduce the availability of amino acids used for synthesis of other compounds. Finally, either the expressed protein or altered levels of other proteins or metabolites might have antinutritional effects[32].

In general, to assess the effects (if any) of a novel protein expressed in a recombinant-DNA plant a number of key parameters are selected: (i) prior history of safe use of the protein in food; (ii) knowledge of the mode of action e.g. enzyme function; (iii) digestibility of the protein in *in vitro* models; (iv) absence of amino acid sequence similarity to sequences in available databases of known mammalian protein toxins and protein allergens or pharmacologically active proteins; (v) predictable levels of expression of the newly introduced protein.

For recombinant-DNA plants that were not developed to have intentionally altered nutritional value, the aim of the nutritional evaluation is to demonstrate that there has been no unintentional change in the levels of key nutrients, natural toxicants or antinutrients, or in the bioavailability of nutrients. In this case, food substitution using products from the recombinant-DNA plants should not adversely affect the health or nutritional status of the consumer. Implications for the population as a whole and for specific subgroups (e.g. children and the elderly) should be considered.

Nevertheless, information on the composition of many plant species is limited, especially with regard to the antinutrient and natural toxin profiles. Because of this, compositional analysis is often hampered when used as a screening method for unintended effects of genetic modification. It is necessary to develop alternative analytical methods that are more informative in these cases. More advanced methodologies, such as mRNA fingerprinting and metabolomic analysis, are being developed but remain to be validated as alternative means of detecting important differences in gene expression and establishing the toxicological significance of the alteration.

Metabolites are dependent on the nutrient profile of a food, which is assessed using the following steps: compositional analysis, morphological and physiological analysis in the form of *in vitro* tests, animal studies and clinical analysis through human studies. Because a broad

31 Key nutrients or key antinutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates as nutrients or enzyme inhibitors as antinutrients) or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased, selenium in wheat) and allergens.

32 Changes in gene expression will also occur when conventional breeding methods are used. Unintended changes in plant composition have been argued to be less frequent in recombinant-DNA plants because only a limited number of genes are transferred during the genetic modification process.

selection is made of nutritionally relevant compounds, and known antinutritional and toxic compounds, the targeted analytical approach, i.e. measuring the content of single substances, offers the assurance that unintentional alterations in plant metabolic pathways will be detected. Where changes in plant metabolites raise significant safety concerns, it may be possible to test their safety individually, or when they are present as a component of the food derived from the recombinant-DNA plant.

The basic information required for recombinant-DNA plants includes measurement of various carbohydrates, proteins, fats, energy and water (Greenfield and Southgate, 1996). Data on key vitamins and minerals are required where deficiencies are a cause of disease and for nutritionally modified foods.

The measurement of carbohydrates (McCleary *et al.*, 2006) can be conducted by various means: (i) analytical methods, which measure total starch, resistant starch and dietary fibre; (ii) chemical – the enzymatic degradation of polysaccharides or oligosaccharides to basic sugars; (iii) physical methods, which assess the food structure retained or conferred; (iv) an assessment of functional properties, such as whether the product is glycaemic, digestible, fermentable, etc.

Amino acid analyses are used to determine the protein content of novel foods. This can be achieved by using the Kjeldahl method (or similar) (Association of Official Analytical Chemistry, 2002), which in principle measures the nitrogen content in order to determine the protein content[33]. Alternatively, relying on their structure, proteins can be hydrolysed to their component amino acids, which can then be measured by ion-exchange, gas-liquid or high-performance liquid chromatography. The sum of the amino acids then represents the protein content (by weight) of the food.

Most of the fat in food is in the form of triglycerides. Fats are analysed either as fatty acids and the result expressed as triglycerides or are measured as the fraction of the food that is soluble in lipid solvents.

Food processing

Processing methods can cause a significant variation in the nutrient content of a food compared with the nutrient profile of the crop as it was grown in a field (Morris *et al.*, 2004).

Modern separation techniques, such as milling, centrifugation, and pressing, change the nutritional content of food, preserving certain nutrients while removing others. Because of reduced nutritional value, processed foods are often "enriched" or "fortified" with some of the most critical nutrients (usually certain vitamins) that were lost during processing. Nonetheless, processed foods tend to have an inferior nutritional profile to whole, fresh foods, with respect to the content of sugar, starch, potassium/sodium, vitamins, fibre, and intact, unoxidized (essential) fatty acids. In addition, processed foods often contain potentially harmful substances such as oxidized fats and trans-fatty acids.

Heating techniques may reduce the content of many heat-labile nutrients such as certain vitamins and phytochemicals, and possibly other as yet undiscovered substances. For example, boiling a potato can cause a significant amount of the potato's B and C vitamins to be lost through an osmotic reaction between the potato and the boiling water. Similar losses occur when food is roasted or fried in oil. The actual nutrient losses observed are affected by many factors such as food type, cooking time and temperature.

CODEX GUIDELINE PARAGRAPH 47. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.

33 This approach is based on two assumptions: that dietary carbohydrates and fats do not contain nitrogen and that nearly all of the nitrogen in the diet is present as amino acids in proteins.

Nutritional modification

For recombinant-DNA plants that were intentionally developed to have altered nutrients, the aim of the nutritional evaluation is to demonstrate that there are no additional unintentional changes in the levels of nutrients, including changes in the bioavailability of these nutrients.

The approach to the safety assessment of products with intentionally modified nutrient profiles is fundamentally the same as for the first generation of recombinant-DNA plants (OECD, 2001). However, the compositional differences between these products and their conventional counterparts are likely to be greater, thus increasing the potential for unintended effects. In essence, the utility of current methods for assessing the safety of recombinant-DNA plants may be found to be limited, due to the fact that the nutritionally modified crops will not be substantially equivalent to their conventional counterparts and will share fewer compositional values for comparison.

Nutritionally modified products may be produced to address a specific dietary or nutritional need. The safety assessment, however, must consider not only the target group but also groups in the population that may be at risk, thus recognizing the presence of population diversity. This requires validated data on food consumption patterns, nutrient intake and in some instances the nutritional status of a population or target group. The safety assessment of a nutritionally modified food must be considered in the context of a total diet.

Due to the potential for broad changes in nutrient levels and interactions with other nutrients, and unexpected effects, it may be necessary in certain instances to undertake feeding

CODEX GUIDELINE PARAGRAPH 48. The assessment of possible compositional changes to key nutrients, which should be conducted for all recombinant-DNA plants, has already been addressed under 'Compositional analyses of key components'. However, foods derived from recombinant-DNA plants that have undergone modification to intentionally alter nutritional quality or functionality should be subjected to additional nutritional assessment to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

CODEX GUIDELINE PARAGRAPH 49. Information about the known patterns of use and consumption of a food, and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA plant. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.

CODEX GUIDELINE PARAGRAPH 50. The use of plant breeding, including in *vitro* nucleic acid techniques, to change nutrient levels in crops can result in broad changes to the nutrient profile in two ways. The intended modification in plant constituents could change the overall nutrient profile of the plant product and this change could affect the nutritional status of individuals consuming the food. Unexpected alterations in nutrients could have the same effect. Although the recombinant-DNA plant components may be individually assessed as safe, the impact of the change on the overall nutrient profile should be determined.

CODEX GUIDELINE PARAGRAPH 51. When the modification results in a food product, such as vegetable oil, with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods or food components whose nutritional composition is closer to that of the food derived from recombinant-DNA plant) as appropriate comparators to assess the nutritional impact of the food.

CODEX GUIDELINE PARAGRAPH 52. Because of geographical and cultural variation in food consumption patterns, nutritional changes to a specific food may have a greater impact in some geographical areas or in some cultural population than in others. Some food plants serve as the major source of a particular nutrient in some populations. The nutrient and the populations affected should be identified.

CODEX GUIDELINE PARAGRAPH 53. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.

studies in other animals to determine the outcomes that result from changes in nutrient profiles and nutrient bioavailability.

New analytical methods

Improved methodologies and more sensitive techniques allow detection of unintended alterations in the composition of foods in a way that was once not possible. The application of profiling methods such as DNA/RNA microarray technology, proteomics, gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to nuclear magnetic resonance (HPLC-NMR) has the potential to provide indications of changes at the level of mRNA expression, protein production and/or at the level of metabolism without prior knowledge of specific changes in plant constituents.

The utility and applicability of these non-targeted techniques for risk assessment needs further exploration, in particular with respect to establishing and validating the relevance to food safety of the observed changes. One of the major difficulties is to distinguish between natural variations and variations that have resulted from genetic modification. It is essential that databases of plant constituent profiles under different conditions contain the full range of values of each measured parameter under a wide range of environmental, genetic, and development conditions. This information would need to be correlated with the presence or absence of associated food safety hazards.

Profiling methods are not yet suitable for routine risk assessment purposes, and further development and validation are needed. A more promising application of these methods may currently lie in a hypothesis-driven analysis of relevant categories of compounds that may be altered. Thus profiling methods are not aimed at replacing conventional single compound analyses, but may be useful, when validated, to confirm and supplement other data.

References

Association of Official Analytical Chemistry. 2002. Official methods of analysis. 2002. Washington, DC, Association of Official Analytical Chemistry.

Greenfield, H. & Southgate, D.A.T. 2003. *Food composition data: production, management and use*, 2nd edition.

McCleary, B.V., Charnock, S.J., Rossiter, P.C., O'Shea, M.F., Power, A.M. & Lloyd, R.M. 2006. Measurement of carbohydrates in grain, feed and food. *J. Sci. Food Agric.*, 86: 1648-1661.

Morris, A., Barnett, A. & Burrows, O.-J. 2004. Effect of processing on nutrient content of foods. *CAJANUS*, 37: 160-164.

OECD. 2001. *Report of the OECD workshop on the nutritional assessment of novel foods and feeds*. Ottawa, Organisation for Economic Co-operation and Development. Feb. 2001. Source: ENV/JM/MONO (2002)6.

Additional resources

International Life Sciences Institute (ILSI). Crop Composition Database. A comprehensive online crop composition database that provides up-to-date information on the natural variability in the composition of conventional crops and provides a reference for comparing the composition of new crop varieties, including those developed through biotechnology. http://www.cropcomposition.org/

See also: **ILSI.** 2003. *Best practices for the conduct of animal studies to evaluate crops genetically modified for input traits*. Washington, DC, ILSI Press. http://www.ilsi.org/AboutILSI/IFBIC/BESTPRACTICES.htm

FAO INFOODS. The International Food Data Systems Project (INFOODS) is a comprehensive effort, begun within the UN University's Food and Nutrition Programme, to improve data on the nutrient composition of foods from all parts of the world, with the goal of ensuring that adequate and reliable data can be obtained and interpreted properly worldwide. http://www.fao.org/infoods/directory_en.stm

OECD. 1998. *Report of the OECD workshop on the toxicological and nutritional testing of novel foods*. Paris, Organization for Economic Co-operation and Development (OECD).

USDA National Nutrient Database for Standard Reference. The Nutrient Data Laboratory (NDL) has the responsibility to develop the USDA's National Nutrient Database for Standard Reference, the foundation of most food and nutrition databases in the United States, which is used in determining food policy, research and nutrition monitoring. http://www.nal.usda.gov/fnic/foodcomp/search ●

9. Perspectives on safety assessment of foods derived from the next generation of recombinant-DNA plants

Introduction

In recent years, the genetic alterations in new plant varieties under development have become more complex, with more genes involved and with an increasing tendency to alter existing metabolic pathways or even introduce new ones. These "second generation" recombinant-DNA plants have been deliberately modified to express novel traits to enhance nutrition and health (e.g. increased vitamin levels) or to improve livestock feed. Unlike the first generation of recombinant-DNA crops, which were not intended to have altered nutritional properties and whose single-gene traits were relatively straightforward to assess for safety, these second generation products may be intentionally designed not to be substantially equivalent to their conventional counterparts. This may introduce new challenges for those tasked with assessing the safety of foods and feedstuffs derived from these recombinant-DNA plants as there may be no conventional comparator against which the foods derived from the recombinant-DNA plants can be measured.

The next generation of recombinant-DNA plants is likely to be genetically more complex (and may blur the boundary between foods and therapeutics e.g. nutraceuticals, edible vaccines and biopharmaceuticals). This will make the application of the concept of substantial equivalence less appropriate, and the safety assessment of such products is likely to depend on additional approaches to assessment and parallel improvements in the understanding of the relationship between plant composition and health impacts. Ensuring that the safety assessment takes into account the dietary needs and consumption patterns of potentially affected (sub)populations will be vital.

It is anticipated that GM food products that have been modified to be significantly different from their conventional counterparts will receive different, if not greater, scrutiny than those GM foods that have been approved by regulatory authorities to date. New analytical methods for predicting and assessing these differences are being considered (reviewed by Kuiper and Kleter, 2003). The utility of these methods is constrained at present by the fact that insufficient data are available to indicate if statistically significant differences that may be identified using profiling methods such as DNA or RNA microarrays or proteomics are biologically relevant from a safety standpoint.

Internationally, few attempts have been made to examine how best to assess the safety of GM foods with enhanced nutritional or health benefits. The International Life Sciences Institute has published a document that provides the scientific underpinnings and recommendations for assessing the safety and nutritional effects of crops with improved nutritional qualities (the document does not address GM foods that offer potential health benefits). It includes terms and definitions for describing such products, identifies the key safety and nutritional challenges, and introduces potential approaches and methods to address those challenges (ILSI, 2004). A more recent initiative has been undertaken by the Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology. In 2005, the Task Force agreed to initiate new work to develop an annex to its 2003 Guideline (see Appendix 2). The annex will elaborate on the guidance provided in the 2003 Guideline.

General principles for the addition of essential nutrients to foods

The Codex Alimentarius Commission (CAC, 2007) provides the guidance for the maintenance or improvement of the overall nutritional quality of foods through the addition of essential nutrients for the purposes of fortification, restoration and nutritional equivalence. The General Principles also address the addition of essential nutrients to special purpose foods to ensure an adequate and appropriate nutrient content. The General Principles aim to prevent the indiscriminate addition of essential nutrients to foods, thereby decreasing the risk of health hazard due to nutrient excesses, deficits or imbalances. The Codex introduced these general principles in 1987 with subsequent amendments. Internationally, there is increased understanding of nutrient enhanced or health promoting foods. It is however a scientifically more involved field of research requiring a different treatment compared with providing a crop variety with increased resistance to a disease, insect pest or herbicide, even when biotechnological tools are utilized for the purpose.

The general principles review by CAC (2007) focused on:

1. new methods of achieving addition or enhancement of the levels of essential nutrients in foods, including biofortification;
2. the need for additional approaches to controlling the addition of essential nutrients to foods, including discretionary fortification;
3. the addition to foods of bioactive substances.

Biofortification

The Codex review (2007) defines biofortification as the indirect addition of essential nutrients or 'other substances' to foods for the purpose of nutritional enhancement or health enhancement. In addition to direct addition of the nutrient to foods at the time of processing, it is possible indirectly to add the nutrients at an earlier point of food production. Genetic transformation using recombinant-DNA technology is one such tool, using which the plant or animal is enabled to produce the additional nutrient e.g. an enhanced beta-carotene level in rice (see Box 9.1).

Box 9.1. Golden rice

An example of this new generation of recombinant-DNA plants is the development of "Golden Rice" in an international network involving the European Union, India, the Philippines, Vietnam and Bangladesh (http://www.goldenrice.org). Dietary micronutrient deficiencies, such as the lack of vitamin A, are a major source of morbidity (increased susceptibility to disease) and mortality worldwide. This deficiency affects particularly children, impairing their immune systems and normal development, causing disease and ultimately death. The best way to avoid micronutrient deficiencies is by way of a varied diet, rich in vegetables, fruits and animal products. According to the WHO, dietary vitamin A deficiency (VAD) causes some 250 000 to 500 000 children to go blind each year. For people who live below the poverty line in many parts of the world, the common foods consumed, such as rice, need to provide vitamin A. However, rice plants produce β-carotene (provitamin A) in green tissues only and not in the endosperm (the edible part of the seed). In Golden Rice, genes have been inserted into the rice genome by genetic engineering that account for the production and accumulation of β-carotene in the grains. The intensity of the golden colour is an indicator of the concentration of β-carotene in the endosperm. After the concept was proved in 1999, new rice lines with higher _-β-carotene content have been generated and are undergoing field trials. The risk analysis and regulation processes are being followed by adhering to the national system and the Codex guidelines in each country. The goal is to be capable of providing the recommended daily allowance of vitamin A - in the form of β-carotene – in 100–200 g of rice, which corresponds to the daily rice consumption of children in rice-based societies, such as in India, Vietnam or Bangladesh. In other countries, *Golden Rice* could still be a valuable complement to children's diets, thus contributing to the reduction of clinical and subclinical VAD-related diseases. *Golden Rice* is a product that does not create new dependencies or displace traditional cuisine and has the potential to save a large number of people from VAD-related diseases.

Box 9.2. Key features of biosafety considerations for nutritionally enhanced foods

a) Estimation of potential exposure distribution patterns: how to go about determining potential exposure distribution patterns in both target and non-target populations of a country and evaluate the safety of such exposure in vulnerable groups. Although techniques are available to simulate such patterns using modeling, it is felt that there is no substitute for controlled trials and investigations, first in animals, and then in consenting, informed humans. In this perspective, an important social issue needs to be taken care of, which is to label the foods derived from recombinant-DNA in the marketplace to provide a means of identifying the GM foods in epidemiological studies as part of post-release monitoring and risk management.

b) Bioavailability: bioavailability analysis should be incorporated into regulatory reviews of all recombinant-DNA plants developed for enhanced nutrition or health. Bioavailability studies using cell cultures have been recommended before feeding trials are taken up and employ radiolabelled compounds to trace the fate of the nutrient upon metabolism in a cell (Wood and Tamura, 2001).

c) Upper limits of safe intake: the need to determine upper limits of safe intake for the nutrient or bioactive substance is essential to eliminate any risk associated with excessive intake of the food. Upper limits of foods containing enhanced nutrients have to be determined for each targeted nutrient as different nutrients can have different upper limits for their safe intake in human beings. Recombinant-DNA plants with modifications of nutrients need to be clearly identified and efforts taken to prevent their use without informed consent. Safe upper limits of ingestion need to be established using the pure form of the targeted nutrient followed by the edible form of the foodstuffs, first in animals then in human volunteers.

d) Stability testing of novel proteins introduced into the recombinant-DNA derived crop as a foodstuff needs to be undertaken because the novel products may create unexpected toxic by-products, especially when the primary plant product is processed into different forms and preparations. The behaviour of these proteins, if unknown from other sources of food, must be subjected to testing in processing as well as storage, in addition to the toxicity testing of the product.

Based on the models developed in Canada (Health Canada, 2005), by the European Commission (EC, 2006), and by Rasmussen *et al.* (2006), an attempt is being made to identify threshold limits for enhanced nutrients so that the risk of indiscriminate addition of essential nutrients is reduced and their effects on health can be evaluated. Similarly, there is a need for further research to identify nutrient-wise (case by case) the minimum levels of addition of nutrients to a biofortified food so that its desired consequence is realized beyond the discernible effect, to enable properly informed labelling of the product.

The Independent Science Panel, launched in 2003 in the United Kingdom[34], has discussed the issue of biosafety of nutritionally enhanced transgenic foods in response to the questionnaire developed by the Codex targeting recombinant-DNA derived foods aimed at nutritional or health benefits. Some key features of the biosafety considerations for nutritionally enhanced foods and crops are described in Box 9.2.

References

Codex Alimentarius. 2006. *Report of the fifth session of the Codex ad hoc intergovernmental task force on foods derived from biotechnology (Alinorm 06/29/34)*. Rome, Codex Alimentarius.

Codex Alimentarius Commission (CAC). 2007. *Report of the Working Group on the Proposed Draft Annex to the Codex Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants: Food safety assessment of foods derived from recombinant-DNA plants modified for nutritional or health benefits*. Rome, CL 2007/18-FBT.

European Commission (EC). 2006. *Discussion paper on the setting of maximum and minimum amounts for vitamins and minerals in foodstuffs*. Brussels, EC Health & Consumer Protection Directorate E.

Health Canada. 2005. *Addition of vitamins and minerals to foods - Health Canada's proposed policy and implementation plans*. http://www.hc-sc.gc.ca/fn-an/consultation/init/summary_report_vitamins-rapport_sommaire_vitamines_e.html

34 http://www.indsp.org/ISPMembers.php

International Life Sciences Institute (ILSI). 2004. Nutritional and safety assessment of foods and feeds nutritionally improved through biotechnology. *Comp. Rev. Food Sci. Food Safety*, 3: 38-104.

Kuiper, H. & Kleter, G. 2003. The scientific basis for risk assessment and regulation of genetically modified foods. *Food Sci. Tech.*, 14: 277-293.

Rasmussen, S.E., Andersen, N.L., Dragsted, L.O. & Larsen, J.C. 2006. A safe strategy for addition of vitamins and minerals to foods. *Eur. J. Nutr.*, 45: 123-135.

Wood, R.J. & Tamura, T. 2001. Methodological issues in assessing bioavailability of nutrients and other bioactive substances in dietary supplements: summary of workshop discussion. *J. Nutr.*, 131(4 Suppl): 1396S-1398S ●

10. Risk communication among stakeholders

Introduction

Risk communication is one of the three distinct but closely linked components of risk analysis as defined by the Codex Alimentarius Commission[35]. It is "the interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions". Along with risk assessment and risk management, risk communication is integral to the overall risk analysis of a food derived from recombinant-DNA plants. Risk communication is the science of understanding scientific and technological risk and how it is communicated within a sociopolitical structure (Powell, 2000).

The processes of assessing the risks involved, and the methods of managing them whilst focusing on health and the safety of the environment, need to be communicated in a simple comprehensive manner without getting into the depths of the technological details involved. It is useful to make it clear to the stakeholder that if a GM crop has a bacterial gene for a specific protein, it does not mean that the transformed crop will be harbouring the bacterium itself, but it only means that the crop now has the capability of producing the new protein from within its own physiology using the gene that was originally present in the bacterium. Once this is established, the communication details should focus on the various regulatory processes involved in ensuring the safe deployment of the technology and its benefits to the end users.

35 *Working principles for risk analysis for application in the framework of the Codex Alimentarius* (adopted by the 26th Session of the Codex Alimentarius Commission, 2003; Codex Alimentarius Commission Procedural Manual; 13th edition)

Box 10.1. Risk communication in the process of risk analysis

1. promote awareness and understanding of the specific issues under consideration during the risk analysis;
2. promote consistency and transparency in formulating risk management options/recommendations;
3. provide a sound basis for understanding the risk management decisions proposed;
4. improve the overall effectiveness and efficiency of the risk analysis;
5. strengthen the working relationships among participants;
6. foster public understanding of the process, so as to enhance trust and confidence in the safety of the food supply;
7. promote the appropriate involvement of all interested parties;
8. exchange information in relation to the concerns of interested parties about the risks associated with food.

Essential features of risk communication

The Codex Alimentarius Commission (2003) lists the characteristics that should be included in risk communication in the process of risk analysis (Box 10.1).

The major function of risk communication should be to ensure that all information and opinions required for effective risk management are incorporated into the decision-making process. It should include a transparent explanation of the risk assessment policy and of the assessment of risk, including the uncertainty. The need for specific standards or related texts and the procedures followed to determine them, including how the uncertainty was dealt with, should also be clearly explained. It should indicate any constraints, uncertainties, assumptions and their impact on the risk analysis, and minority opinions that have been expressed in the course of the risk assessment. However, even though it is expected to be transparent and accessible to all interested parties, if there are legitimate concerns to

preserve confidentiality, these should be respected while information on the risk analysis should be made available.

Risk communication is an important part of the biosafety procedures that ensure public acceptance of food derived from recombinant-DNA plants. To communicate and interact with the public at large about the specific risks and the actions taken to alleviate them before the recombinant-DNA crop reaches the field or the derived food reaches the markets is a crucial step in reassuring the stakeholders. It is also a mechanism that builds confidence among the stakeholders in a gradual manner, moving along with the different phases of the development of the recombinant-DNA plant and the foods derived from it. In the absence of this channel, a void gets created leading to the stakeholders not being made aware of the efforts taken by the regulators to reduce the risks assessed with the technology. This also encourages the spread of fictitious stories from not fully informed individuals to others, along with their own potentially misleading messages.

Media coverage of food derived from recombinant-DNA plants can become polarized into safety versus risk; science moving forward versus science out of control; competitiveness versus safety (Powell and Leiss, 1997). Media analysis is a tool used to help understand the formation of public opinion and to look at what people are saying and what they are being told. This reliance on the media helps to define the public's sense of reality (Nelkin, 1987) and their perceptions of risks or benefits.

Risk communication can be divided into two major components: technical components that generally comprise the scientific hazards evaluated in the risk assessment and the management options arising out of the assessment, and non-technical components that include the administrative protocols, and the cultural and ethical issues in society as dealt with by the regulatory agencies during the process of risk analysis.

Regulatory risk communication

Regulatory risk communication starts primarily by keeping the stakeholder groups (the whole food chain involving scientist, farmer, trader, processor, product developer, market player [retailer] and consumer) informed of the upcoming technology as soon as the technology development project for a particular crop is approved by an institution. From this stage onwards, methods need to be devised for comprehensible transmission of information at various stages of product development until it reaches the markets, so that the primary stakeholder at each stage is taken into confidence.

It is important that only accurate information should be given, as risk communication tends to influence psychological and cultural beliefs. Assessment of the scientific risks must be coupled with appropriate research-based risk management and communication activities to provide consumers, the media and others with a balanced, science-based assessment of both

CODEX PRINCIPLES PARAGRAPH 22. Effective risk communication is essential at all phases of risk assessment and risk management. It is an interactive process involving all interested parties, including government, industry, academia, media and consumers.

CODEX PRINCIPLES PARAGRAPH 23. Risk communication should include transparent safety assessment and risk management decision-making processes. These processes should be fully documented at all stages and open to public scrutiny, whilst respecting legitimate concerns to safeguard the confidentiality of commercial and industrial information. In particular, reports prepared on the safety assessments and other aspects of the decision-making process should be made available to all interested parties.

CODEX PRINCIPLES PARAGRAPH 24. Effective risk communication should include responsive consultation processes. Consultation processes should be interactive. The views of all interested parties should be sought and relevant food safety and nutritional issues that are raised during consultation should be addressed during the risk analysis process.

the potential benefits and the risks of a particular technology, and to positively impact the development of public policy. The challenge is to incorporate public perceptions into policy development without abdicating the leadership role of science.

Risk communication is addressed in the Codex Principles for the Risk Analysis of Foods Derived from Modern Biotechnology (see Appendix 1) as follows.

Risk communication is used to explain both how and why decisions are taken. It specifically acknowledges any concerns raised by stakeholders, including the public, and explains how these concerns have been addressed. This captures the reality that risk communication is an iterative exchange between interested and affected parties that primarily, but not exclusively, focuses on risks. In practice, because of the wide diversity of stakeholders involved in agricultural biotechnology, risk communication is largely a non-technical dialogue about both real and perceived risks.

It is widely recognized that more could – and should – be done to make information concerning the safety assessment of novel foods available to the public. This has become more important with increased consumer interest in the safety of food derived from recombinant-DNA plants. The OECD countries and intergovernmental organizations are looking for new ways to share their experiences. They are promoting information dissemination and sound understanding of the safety issues on the part of consumers (OECD, 2000). A number of countries have adopted measures concerned with sharing information on the safety assessment of GM foods with the public. These include:

a. inviting public comments on reports containing safety evaluations by scientific assessment bodies;
b. disclosure of data used in safety assessments to support applications;
c. publication of results of meetings of safety assessment bodies.

Regulatory authorities are actively involving, and consulting, the public with regard to food safety and regulation. Some authorities have a policy of full disclosure of the information contained in applications (except for confidential commercial information). The Internet is increasingly used to make information on safety assessment and approval procedures available to the public. It is a good source of information on crops and other foods that have been approved. Some countries are exploring the potential of the Internet to make details of applications more widely available, in order to make the assessment process as open, transparent and inclusive as possible.

The OECD's BioTrack Online site (http://www.oecd.org/ehs/service.htm) is a valuable source of information on regulatory developments in Member countries. It includes information on responsible ministries or agencies, and details of laws, regulations and guidelines. There are also two important databases, one of products that have been commercialized, and the other of field trials of GM crops that have taken place in OECD countries.

Risk communication as a two-way process

Regulatory risk communication deals with providing information about the regulatory framework and processes designed to assess and manage risk, such as policy development, application processes, stakeholder participation, product-specific decisions, and access to the information that is used to inform regulatory decision-making. In order to avoid real or perceived conflicts of interest many regulatory agencies undertake only regulatory risk communication activities and leave more technology- or product-focused communication efforts to other stakeholder groups. As much effort should be put into gathering input and feedback as into giving out information.

To be effective, regulatory risk communicators need to devise appropriate mechanisms to receive feedback, analyse it and use the information to revise and improve their communication outreach. Obtaining feedback and input from stakeholders enables regulators and risk assessors

Box 10.2. Useful considerations in risk communication

Know the audience
Before formulating risk communication messages, the audience should be analysed to understand their motivations and opinions. Beyond knowing in general who the audience is, it is necessary actually to get to know them as groups, and ideally as individuals, to understand their concerns and feelings and to maintain an open channel of communication with them. Listening to all interested parties is an important part of risk communication.

Involve the scientific experts
Scientific experts, in their capacity as risk assessors, must be able to explain the concepts and processes of risk assessment. They need to be able to explain the results of their assessment and the scientific data, assumptions and subjective judgements upon which it is based, so that risk managers and other interested parties clearly understand the risk. They must also be able to communicate clearly what they know and what they do not know, and to explain the uncertainties related to the risk assessment process. In turn, the risk managers must be able to explain how the risk management decisions have been arrived at.

Establish expertise in communication
Successful risk communication requires expertise in conveying understandable and usable information to all interested parties. Risk managers and technical experts may not have the time or the skill to perform complex risk communication tasks, such as responding to the needs of the various audiences (public, industry, media, etc.) and preparing effective messages. People with expertise in risk communication should therefore be involved as early as possible in the process. This expertise will probably have to be developed by training and experience.

Be a credible source of information
Information from credible sources is more likely to influence the public perception of a risk than is information from sources that lack this attribute. The credibility accorded to a source by a target audience may vary according to the nature of the hazard, culture, social and economic status, and other factors. If consistent messages are received from multiple sources then the credibility of the message is reinforced. Factors determining source credibility include recognized competence or expertise, trustworthiness, fairness and lack of bias. For example, the terms that consumers have associated with high credibility include factual, knowledgeable, expert, public welfare, responsible, truthful and good "track record". Trust and credibility must be nurtured, and it can be eroded or lost through ineffective or inappropriate communication. In studies, consumers have indicated that distrust and low credibility result from exaggeration, distortion and perceived vested interest.

Effective communications acknowledge current issues and problems, are open in their content and approach, and are timely. Timeliness of the message is most important because many controversies become focused on the question "why didn't you tell us sooner?", rather than on the risk itself. Omissions, distortions and self-serving statements will damage credibility in the longer term.

Share responsibility
Regulatory agencies of governments at the national, regional and local levels have a fundamental responsibility for risk communication. The public expects the government to play a leading role in managing public health risks. This is true when the risk management decision involves regulatory or voluntary controls, and is even true when the government decision is to take no action. In the latter event, communication is still essential to provide the reasons why taking no action is the best option. In order to understand public concerns and to ensure that risk management decisions respond to those concerns in appropriate ways, the government needs to determine what the public knows about the risks and what the public thinks of the various options being considered to manage those risks.

The media play an essential role in the communication process and therefore share these responsibilities. Communication on immediate risks involving human health, particularly when there is a potential for serious health consequences, such as with food-borne illnesses, cannot be treated in the same manner as less immediate food safety concerns. Industry also has a responsibility for risk communication, especially when the risk is as a result of their products or processes. All parties involved in the risk communication process (e.g. government, industry, media) have joint responsibilities for the outcome of that communication, even though their individual roles may differ. Because science must be the basis for decision-making, all parties involved in the communication process should know the basic principles and data supporting the risk assessment and the policies underlying the resulting risk management decisions.

Differentiate between science and value judgement
It is essential to separate "facts" from "values" in considering risk management options. At a practical level, it is useful to report the facts that are known at the time as well as the uncertainties that are involved in the risk management decisions being proposed or implemented. The risk communicator bears the responsibility to explain what is known as fact and where the limits of this knowledge begin and end. Value judgements are involved in the concept of acceptable levels of risk. Consequently, risk communicators should be able to justify the level of acceptable risk to the public. Many people take the term "safe food" to mean food with zero risk, but zero risk is often unattainable. In practice, "safe food" usually means food that is "safe enough". Making this clear is an important function of risk communication.

Assure transparency
For the public to accept the risk analysis process and its outcomes, the process must be transparent. While respecting legitimate concerns to preserve confidentiality (e.g. proprietary information or data), transparency in risk analysis consists of having the process open and available for scrutiny by interested parties. Effective two-way communication between risk managers and the public and other interested parties is both an essential part of risk management and a key to achieving transparency.

to identify and address stakeholder concerns. Often the best route for information dissemination involves strengthening existing information channels. For example, if governments publish progress updates in local newspapers, mechanisms to use this for agricultural biotechnology risk communication may be best in the short term. However, if governments rely only on mechanisms such as "Government Gazettes", which are poorly distributed, to inform the public then attention needs to be paid to alternative ways of disseminating information to and receiving it from the target groups.

Credibility is often built into a communication process by providing technical reviews of the process in simple language. For example, reviews can be commissioned that explain the science and technology involved in the process and the regulatory procedures involved (Beever and Kemp, 2000).

Different audiences or stakeholder groups have different needs and so it is important to understand an audience well before designing communication for them. Identifying an audience's needs, concerns, knowledge level, opinions and preferred mechanisms for communicating through consultation supports the development of a communication style that will be effective.

The type of audience should also be carefully considered when selecting the best communicators. Effective communicators need to be credible and trusted, and different people may be required for different target groups. In addition communicators need to have excellent language and listening skills. In general, the credibility of communicators depends on cultural norms and differs from society to society and between sectors.

Two targeted questions that need to be answered during risk communication are: "are foods from recombinant-DNA plants safe?" and "what foods have been genetically modified?". This raises the issue of choice and knowing what foods from recombinant-DNA plants may be in the marketplace. In order to address these questions, regulatory authorities typically make information available about the national regulatory framework that identifies the competent authorities; details the regulatory requirements for the different stages in product development (e.g. research and development, confined or experimental field testing, and premarket safety assessments); explains how safety assessments are conducted, and clearly indicates how decisions are made, including opportunities for public participation in decision-making and the factors taken into account by decision-makers. The feedback is also put within a time frame so that any additional information or clarification can be provided to interested parties.

Additionally, many regulatory authorities publish product-specific decision summaries that provide information about specific transgenic events.

The report of a joint FAO/WHO Expert Consultation on the application of risk communication to food standards and safety matters provides a helpful summary of principles for risk communication that are applicable to those involved in communicating about the regulation and safety assessment of foods from recombinant-DNA plants[36].

Risk communication in safety assessment

Although most countries attempt to provide complete and clear information on the foods derived from recombinant-DNA plants, the information itself is often found to be too complex and multidisciplinary in nature to be understood fully by the public without bias or ambiguity. The challenge is to present the material in a suitable form for different audiences without compromising the accuracy of the information. It is necessary to make the message as communicative as possible to enable the consumer to make an informed choice on accepting the food derived from recombinant-DNA plants with reference to the risks associated with it. The Canadian Biotechnology Advisory Committee (CBAC, 2002) considered the options listed below.

a. **Creation of better information about the regulatory system.** An initial step may be to improve the description and communication of information about the Canadian food regulatory

[36] FAO. 1999. *The application of risk communication to food standards and safety matters.* FAO Food and Nutrition Paper 70. Rome, Food and Agriculture Organization. http://www.fao.org/docrep/005/x1271e/X1271E00.HTM

system for GM and other novel foods, and to ensure that the material provided is complete, understandable and easily retrievable. A variety of media (for example, the Internet, booklets, articles) could be used to make the information more widely available. The material could be presented with various levels of complexity to be helpful to different readers.

b. **Creation of a centralized information body.** A centralized body for consumer information on food biotechnology could provide information on food production, GM foods and other novel food biotechnology, relevant laws and regulations, scientific knowledge, perspectives on ethical and social issues, ongoing research and activities, and how to contribute to government-related activities. In addition to discussing the traditional foods and plant-breeding practices, an attempt should be made to provide a meaningful description of the benefits, risks and uncertainties associated with different types of foods.

c. **Increase public awareness and engagement.** In addition to the above options, a proactive communications programme may be useful for increasing public awareness. Opportunities for Canadians to comment on various aspects of GM foods could be provided through public dialogue sessions.

The Biotechnology Consortium of India Limited (BCIL) is another such communication portal and is a unique combination of public–private partnership providing all the technical information and social concerns with respect to biosafety assessment on recombinant-DNA research and commercial activities. Developed on the pattern of the biosafety clearing house concept, it also undertakes to conduct workshops in different parts of the country in an open forum involving all stakeholders and regulatory agencies on specific issues (BCIL, 2007). For interested parties, hyperlinks or downloadable access to self-contained reviews may be provided to enable an informed understanding among stakeholders on the safety issues, and effective management strategies.

References

APUA. 2000. *Case study in regulatory issues connected with genetically engineered foods: genetically engineered corn runs into regulatory problems in Europe*. A joint project of the University of Illinois, Urbana and the Alliance for the Prudent Use of Antibiotics (Tufts University) to develop a network to monitor resistance in commensal bacteria. 22 pp. http://www.agbios.com/docroot/articles/salyersreport.pdf

Beever, D.E. & Kemp, C.F. 2000. Safety issues associated with the DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures. *Nutr. Abstr. Rev. Series B: Livestock Feeds and Feeding*, 70: 175–182.

Biotechnology Consortium of India Limited (BCIL). 2007. http://bcil.nic.in

Canadian Biotechnology Advisory Committee (CBAC). 2002. *Improving the regulation of genetically modified foods and other novel foods in Canada*. Ottawa, Canada. http://cbac-cccb.ca/epic/site/cbac-cccb.nsf/en/ah00186e.html

Codex Alimentarius Commission (CAC). 2003. *Risk analysis policies of CAC*. Twenty-sixth session of CAC. Rome. 30 June-3 July 2003.

Defra. 2001. *Guidance on principles of best practice in the design of genetically modified plants*. Advisory Committee on Releases to the Environment, ACRE, March 2001. http://www.defra.gov.uk/environment/acre/bestprac/consult/guidance/bp/index.htm

European Commission. 2003. *Guidance document for the risk assessment of genetically modified plants and derived food*. Scientific Steering Committee, European Commission. 6–7 March 2003, Brussels. APUA. 2000. Case study in regulatory issues connected with genetically engineered foods: genetically engineered corn runs into regulatory problems in Europe. http://ec.europa.eu/food/fs/sc/ssc/out327_en.pdf

FAO/WHO. 2000. *Safety aspects of genetically modified foods of plant origin. Report of a joint FAO/WHO expert consultation on foods derived from biotechnology*, 29. Food and

Agriculture Organization of the United Nations (FAO), Rome and World Health Organization (WHO). ftp://ftp.fao.org/docrep/nonfao/ae584e/ae584e00.pdf

FAO/WHO. 2001. *FAO/WHO expert consultation on foods derived from biotechnology. Evaluation of allergenicity of genetically modified foods*. Rome, WHO/FAO, January 2001. http://www.fao.org/ag/agn/food/risk_biotech_allergen_en.stm

FAO/WHO. 2002. *Codex ad hoc intergovernmental task force on foods derived from biotechnology, third session*. Joint FAO/WHO food standards programme, Yokohama, Japan, 4–8 March 2002. ftp://ftp.fao.org/codex/alinorm03/Al03_34e.pdf

Kuiper, H.A., Kleter, G.A., Noteborn, H.P.J.M. & Kok, E.J. 2001. Assessment of the food safety issues related to genetically modified foods. *Plant J.*, 27: 503–528. http://www.blackwell-synergy.com/doi/pdf/10.1046/j.1365-313X.2001.01119.x

Nelkin, D. 1987. *Selling science: how the press covers science and technology*. New York, W.H. Freeman and Company.

OECD. Biotech Product Database Web Site, http://webdomino1.oecd.org/ehs/bioprod.nsf .

OECD. Biotrack Online Web Site, http://www.oecd.org/ehs/service.htm.

OECD. Task Force for the Safety of Novel Foods and Feeds Web Site. http://www.oecd.org/document/63/0,2340,en_2649_34391_1905919_1_1_1_1,00.html

OECD. 2000. *Consensus documents for the work on the safety of novel foods and feeds*. Organisation for Economic Co-operation and Development. http://www.oecd.org/document/9/0,3343,en_2649_34391_1812041_1_1_1_1,00.html

OECD. 2000. *Report of the task force for the safety of novel foods and feeds*. Paris, Organisation for Economic Co-operation and Development. 72 pp.

Powell, D. & Leiss, W. 1997. *Mad Cows And Mother's Milk: The Perils of Poor Risk Communication*. Kingston, Canada, McGill-Queen's University Press.

Powell, D.A. 2000. Food safety and the consumer perils of poor risk communication. *Can. J. Anim. Sci.*, 80: 393–404 ●

11. Glossary of terms, links and resources

The following terms frequently appear in dossiers submitted for safety evaluation. For more information on biotechnology-related terminology, see the FAO Glossary of Biotechnology for Food and Agriculture at http://www.fao.org/biotech/index_glossary.asp

Glossary

Adjuvant
An agent mixed with an antigen that enhances the immune response to that antigen or to immunization.

Antisense gene
A gene that produces a transcript (mRNA) that is complementary to the pre-mRNA or mRNA of a normal gene (usually constructed by inverting the coding region relative to the promoter).

Bioavailability
The proportion of a nutrient or administered drug, etc. that can be taken up by an organism in a biologically effective form. For example, some soils high in phosphorus (P) have a low level of P availability because the pH of the soil renders much of the P insoluble.

Biosafety
Refers to the avoidance of risk to human health and safety, and to the conservation of the environment, during the use for research and commerce of infectious or genetically modified organisms.

Biotechnology (modern)
The application of:
1. *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles; or
2. Fusion of cells beyond the taxonomic family that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection (Cartagena Protocol on Biosafety to the Convention on Biological Diversity).

Biotechnology (traditional)
1. Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use (Convention on Biological Diversity).
2. Interpreted in a narrow sense, which considers only the new DNA techniques, molecular biology and reproductive technological applications, biotechnology covers a range of different technologies such as gene manipulation and gene transfer, DNA typing and cloning of plants and animals (FAO statement on biotechnology).

Comparative approach
The comparative approach, previously referred to as substantial equivalence, embodies the concept that GM foods can be assessed to a large extent by comparison to the benchmark of commonly consumed foods already regarded as safe (the traditional or non-modified counterpart). The comparison is usually made at the level of the composition of the food.

Concatemer
A DNA segment made up of repeated sequences linked head to tail.

Concatenation
Combination of two (or more) strings of DNA in a defined order.

Conventional counterpart
A related plant variety, its components and/or products for which there is experience of establishing safety based on common use as food.

Copy number
The number of copies of a particular plasmid per bacterial cell, or copies of a gene per genome.

Dietary exposure
Contact by ingestion between a physical, chemical or biological agent and an organism.

Gene silencing
Gene silencing is a general term describing epigenetic processes of gene regulation and refers to an event of interruption or suppression of the expression of a gene. Genes are regulated at either the transcriptional or post-transcriptional level. Transcriptional gene silencing is the result of histone modifications, creating an environment of heterochromatin around a gene that makes it inaccessible to transcriptional machinery. Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed. The destruction of the mRNA prevents translation to form an active gene product. The term frequently appears in the dossiers often refers to a natural reaction of plants to high levels of foreign gene expression. However, not all foreign gene expression leads to gene silencing. Many factors contribute to gene silencing including the nature and orientation of the foreign transgenes, expression levels and phase of development.

Genetic engineering
Modification of the genotype, and hence the phenotype, by transgenesis, which is the introduction of a gene or genes into animal or plant cells, which leads to the transmission of the input gene (transgene) to successive generations.

Genetically modified foods (GM foods)
Genetically modified (GM) foods are foods produced from genetically modified organisms (GMOs) that have had their genome altered through genetic engineering (e.g. GM corn) or foods that contain ingredients from GMOs (e.g. chocolate containing GM soybeans).

Genetically modified organism (GMO)
An organism that has been transformed by the insertion of one or more transgenes.

Hapten
A small molecule, which by itself is not an antigen, but which as a part of a larger structure when linked to a carrier protein, can serve as an antigenic determinant.

Helper plasmid
A plasmid that provides a function or functions to another plasmid in the same cell.

Immunoglobulin E (IgE)
Class E immunoglobulins (IgE) are highly specialized antibodies that are produced in lymphatic tissue near the respiratory and digestive tracts. Although they make up only 0.001 percent of antibodies, IgE immunoglobulins are involved in virtually every allergic reaction. IgE antibodies dock onto their respective allergen and stimulate the production of substances that cause inflammation. The subsequent immune over-reaction is known as an allergy. Specialized IgE antibodies can be detected in the blood serum of individuals who are sensitive to the respective allergen.

In vitro digestibility assay
Methods are available for determining the digestibility of protein-containing composites, including foods and feed ingredients. The methods comprise incubation of the composite with proteases, followed by determination of the hydrolysed peptide bonds. The methods are suitable for rapid, routine determination of digestibility in food and feed processing plants.

Isogenic parental line
In genetically modified plants, isogenic initial lines mean those non-GM plants from which the GM strains are derived. Thus, the only difference between GM plants and their derivative isogenic line will be those genes that have been transferred transgenically. Evaluating GM plants for possible unexpected effects necessitates comparison with unmodified parental strains. In order to eliminate any possible influence of normal genetic variation between different hereditary lines and varieties, isogenic lines are usually used as a standard for comparison.

Open reading frame (ORF)
A sequence of nucleotides in a DNA molecule that has the potential to encode a peptide or protein. An ORF contains a start triplet (ATG), which is followed by a series of triplets (each of which encodes an amino acid), and ends with a stop codon (TAA, TAG or TGA). The term is generally applied to sequences of DNA fragments for which no function has yet been determined. The number of ORFs provides an estimate of the number of genes transcribed from the DNA sequence.

Outcrossing
A mating between different populations or individuals of the same species that are not closely related. The term "outcrossing" can be used to describe unintended pollination by an outside source of the same crop during hybrid seed production.

Pleiotropy (pleiotropic effects)
The simultaneous effect of a given gene on more than one apparently unrelated trait.

Positional effect
The influence of the location of a gene (particularly a transgene) on its expression and hence on the phenotype.

Post-translational modification
The addition of specific chemical residues to a protein after it has been translated. Common residues are phosphate groups (phosphorylation) and sugars (glycosylation).

Recombinant
A term used in both classical and molecular genetics.
1. In classical genetics: an organism or cell that is the result of meiotic recombination.
2. In molecular genetics: a hybrid molecule made up of DNA obtained from different organisms. Typically used as an adjective, e.g. recombinant-DNA.

Recombinant-DNA
The result of combining DNA fragments from different sources.

Substantial equivalence
Substantial equivalence is a concept, first described in an OECD publication in 1993, which stresses that an assessment of a novel food, in particular one that is genetically modified, should demonstrate that the food is as safe as its traditional counterpart.

Toxicokinetics
The study of the time-dependent processes related to toxicants as they interact with living organisms. It encompasses absorption, distribution, storage, biotransformation and elimination.

Transfer DNA (T-DNA)
The DNA segment of the Ti plasmid, present in pathogenic Agrobacterium tumefaciens, that is transferred to plant cells and inserted into the plant's DNA as part of the infection process. Wild-type T-DNA encodes enzymes that induce the plant to synthesize specific opines that are required for bacterial growth. In engineered T-DNAs, these genes are replaced by one or more transgenes.

Transgene
An isolated gene sequence used to transform an organism. Often, but not always, the transgene has been derived from a different species from that of the recipient.

Weediness
The ability of a plant to colonize a disturbed habitat and compete with cultivated species.

Links and resources

Inter-governmental organizations

Food and Agriculture Organization
The multi-lingual FAO Biotechnology website provides access to updated news and events, documents, an e-mail forum, a glossary, national biotechnology policy documents and other useful information about many aspects of modern biotechnology.
http://www.fao.org/biotech

Codex Alimentarius
The Codex Alimentarius Commission was created in 1963 by FAO and WHO to develop food standards, guidelines and related texts such as codes of practice under the Joint FAO/WHO Food Standards Programme. Related to GM food safety, the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology has published *Principles for the risk analysis of foods derived from modern biotechnology* and *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants*, provided in Appendices 1 and 2 of this monograph. http://www.codexalimentarius.net/web/index_en.jsp

World Health Organization

WHO has been addressing a wide range of issues in the field of biotechnology and human health, including safety evaluation of vaccines produced using biotechnology, human cloning and gene therapy. http://www.who.int/foodsafety/biotech/en/

Organisation for Economic Co-operation and Development

The OECD's programme of work for the Safety of Novel Foods and Feeds is intended to promote international harmonization in the safety assessment and regulation of GM foods and feeds, including the products of modern biotechnology. The OECD's Task Force for the Safety of Novel Foods and Feeds decided at its first session, in 1999, to focus its work on the development of science-based consensus documents, which are mutually acceptable among member countries. These consensus documents contain information for use during the regulatory assessment of a particular food/feed product. In the area of food and feed safety, consensus documents are being published on the nutrients, antinutrients or toxicants, information on the product's use as a food/feed and other relevant information.
http://www.oecd.org/topic/0,2686,en_2649_37437_1_1_1_1_37437,00.html

Biosafety Clearing House

The Biosafety Clearing-House (BCH) is an information exchange mechanism established by the Cartagena Protocol on Biosafety to assist Parties to implement its provisions and to facilitate sharing of information on, and experience with, living modified organisms (LMOs).
http://bch.biodiv.org/

International Centre for Genetic Engineering and Biotechnology

ICGEB offers a rich array of information. The BioSafety web page provides extensive links to international treaties, conventions and meetings, including submissions by member governments. http://www.icgeb.org

United Nations Industrial Development Organization

UNIDO is the only organization that maintains detailed databases of key industrial statistics with worldwide coverage. It has established a network of regional centres providing comprehensive training in biosafety. http://binas.unido.org/wiki/index.php/Main_Page

Institute for Health and Consumer Protection of the Joint Research Center

IHCP is part of the Directorate General JRC and fulfils the JRC's mission in providing scientific support to policies related to health and consumer protection. http://ihcp.jrc.ec.europa.eu/

Some Governmental regulatory web sites related to GM foods

Australia and New Zealand

Food Safety Australia New Zealand (FSANZ).
http://www.foodstandards.gov.au/foodmatters/gmfoods/index.cfm

Canada

Health Canada.
http://www.hc-sc.gc.ca/food-aliment/mh-dm/ofb-bba/nfi-ani/e_novel_foods_and_ingredient.html

European Commission

European Food Safety Authority (EFSA).
http://www.efsa.europa.eu/en/science/gmo.html

India

Department of Biotechnology: Biosafety Rules and Regulations.
http://dbtbiosafety.nic.in/

Japan

Ministry of Health, Labour and Welfare.
http://www.mhlw.go.jp/english/topics/food/index.html

United States

Food and Drug Administration, http://www.cfsan.fda.gov/~lrd/biotechm.html#reg
United States Department of Agriculture, http://www.usda.gov
United States Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, http://www.epa.gov/ ●

Appendices

Relevant Codex documents

Appendix 1.
Principles for the Risk Analysis of Foods Derived from Modern Biotechnology CAC/GL 44-2003

Section 1 – Introduction

1. For many foods, the level of food safety generally accepted by the society reflects the history of their safe consumption by humans. It is recognised that in many cases the knowledge required to manage the risks associated with foods has been acquired in the course of their long history of use. Foods are generally considered safe, provided that care is taken during development, primary production, processing, storage, handling and preparation.

2. The hazards associated with foods are subjected to the risk analysis process of the Codex Alimentarius Commission to assess potential risks and, if necessary, to develop approaches to manage these risks. The conduct of risk analysis is guided by general decisions of the Codex Alimentarius Commission[1] as well as the Codex Working Principles for Risk Analysis[2].

3. While risk analysis has been used over a long period of time to address chemical hazards (*e.g.* residues of pesticides, contaminants, food additives and processing aids), and it is being increasingly used to address microbiological hazards and nutritional factors, the principles were not elaborated specifically for whole foods.

4. The risk analysis approach can, in general terms, be applied to foods including foods derived from modern biotechnology. However, it is recognised that this approach must be modified when applied to a whole food rather than to a discrete hazard that may be present in food.

5. The principles presented in this document should be read in conjunction with the Codex Working Principles for Risk Analysis to which these principles are supplemental.

6. Where appropriate, the results of a risk assessment undertaken by other regulatory authorities may be used to assist in the risk analysis and avoid duplication of work.

Section 2 – Scope and definitions

7. The purpose of these Principles is to provide a framework for undertaking risk analysis on the safety and nutritional aspects of foods derived from modern biotechnology. This document does not address environmental, ethical, moral and socio-economic aspects of the research, development, production and marketing of these foods[3].

8. The definitions below apply to these Principles:

- "Modern Biotechnology" means the application of:
  - i) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or
  - ii) Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombinant barriers and that are not techniques used in traditional breeding and selection[4].
- "Conventional Counterpart" means a related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food[5].

Section 3 – Principles

9. The risk analysis process for foods derived from modern biotechnology should be consistent with the Codex Working Principles for Risk Analysis.

Risk assessment

10. Risk assessment includes a safety assessment, which is designed to identify whether a hazard,

1 These decisions include the Statements of principle concerning the role of science in the Codex decision-making process and the extent to which other factors are taken into account and the Statements of principle relating to the role of food safety risk assessment (Codex Alimentarius Commission Procedural Manual; Thirteenth edition).

2 "Working Principles for Risk Analysis for Application in the Framework of the Codex Alimentarius"(adopted by the 26th Session of the Codex Alimentarius Commission, 2003; Codex Alimentarius Commission Procedural Manual; Thirteenth edition)

3 This document does not address animal feed and animals fed such feed except insofar as these animals have been developed by using modern biotechnology.

4 This definition is taken from the Cartagena Biosafety Protocol under the Convention on Biological Diversity.

5 It is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

nutritional or other safety concern is present, and if present, to gather information on its nature and severity. The safety assessment should include a comparison between the food derived from modern biotechnology and its conventional counterpart focusing on determination of similarities and differences. If a new or altered hazard, nutritional or other safety concern is identified by the safety assessment, the risk associated with it should be characterized to determine its relevance to human health.

11. A safety assessment is characterized by an assessment of a whole food or a component thereof relative to the appropriate conventional counterpart:

A) taking into account both intended and unintended effects;
B) identifying new or altered hazards;
C) identifying changes, relevant to human health, in key nutrients.

12. A pre-market safety assessment should be undertaken following a structured and integrated approach and be performed on a case-by-case basis. The data and information, based on sound science, obtained using appropriate methods and analysed using appropriate statistical techniques, should be of a quality and, as appropriate, of quantity that would withstand scientific peer review.

13. Risk assessment should apply to all relevant aspects of foods derived from modern biotechnology. The risk assessment approach for these foods is based on a consideration of science-based multidisciplinary data and information taking into account the factors mentioned in the accompanying Guidelines[6].

14. Scientific data for risk assessment are generally obtained from a variety of sources, such as the developer of the product, scientific literature, general technical information, independent scientists, regulatory agencies, international bodies and other interested parties. Data should be assessed using appropriate science-based risk assessment methods.

15. Risk assessment should take into account all available scientific data and information derived from different testing procedures, provided that the procedures are scientifically sound and the parameters being measured are comparable.

Risk management

16. Risk management measures for foods derived from modern biotechnology should be proportional to the risk, based on the outcome of the risk assessment and, where relevant, taking into account other legitimate factors in accordance with the general decisions of the Codex Alimentarius Commission[7] as well as the Codex Working Principles for Risk Analysis.

17. It should be recognised that different risk management measures may be capable of achieving the same level of protection with regard to the management of risks associated with safety and nutritional impacts on human health, and therefore would be equivalent.

18. Risk managers should take into account the uncertainties identified in the risk assessment and implement appropriate measures to manage these uncertainties.

19. Risk management measures may include, as appropriate, food labelling[8] conditions for marketing approvals and post-market monitoring.

20. Post-market monitoring may be an appropriate risk management measure in specific circumstances. Its need and utility should be considered, on a case-by-case basis, during risk assessment and its practicability should be considered during risk management. Post-market monitoring may be undertaken for the purpose of:

A) verifying conclusions about the absence or the possible occurrence, impact and significance of potential consumer health effects; and
B) monitoring changes in nutrient intake levels, associated with the introduction of foods likely to significantly alter nutritional status, to determine their human health impact.

21. Specific tools may be needed to facilitate the implementation and enforcement of risk management measures. These may include appropriate analytical methods; reference materials; and, the tracing of products[9] for the purpose of facilitating withdrawal from the market when a risk to human health has been

6 Reference is made to the Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (CAC/GL 45-2003) and the Guideline for the Conduct of Food Safety Assessment of Foods Produced using Recombinant-DNA Microorganisms (CAC/GL 46-2003).

7 See footnote 1.

8 Reference is made to the CCFL in relation to the Proposed Draft Guidelines for the Labelling of Foods and Food Ingredients obtained through certain techniques of genetic modification/genetic engineering at Step 3 of the Codex Elaboration Procedure.

9 It is recognised that there are other applications of product tracing. These applications should be consistent with the provisions of the SPS and TBT Agreements. The application of product tracing to the areas covered by both Agreements is under consideration within Codex on the basis of decisions of 49th Session of Executive Committee.

identified or to support post-market monitoring in circumstances as indicated in paragraph 20.

Risk communication

22. Effective risk communication is essential at all phases of risk assessment and risk management. It is an interactive process involving all interested parties, including government, industry, academia, media and consumers.

23. Risk communication should include transparent safety assessment and risk management decision-making processes. These processes should be fully documented at all stages and open to public scrutiny, whilst respecting legitimate concerns to safeguard the confidentiality of commercial and industrial information. In particular, reports prepared on the safety assessments and other aspects of the decision-making process should be made available to all interested parties.

24. Effective risk communication should include responsive consultation processes. Consultation processes should be interactive. The views of all interested parties should be sought and relevant food safety and nutritional issues that are raised during consultation should be addressed during the risk analysis process.

Consistency

25. A consistent approach should be adopted to characterise and manage safety and nutritional risks associated with foods derived from modern biotechnology. Unjustified differences in the level of risks presented to consumers between these foods and similar conventional foods should be avoided.

26. A transparent and well-defined regulatory framework should be provided in characterising and managing the risks associated with foods derived from modern biotechnology. This should include consistency of data requirements, assessment frameworks, the acceptable level of risk, communication and consultation mechanisms and timely decision processes.

Capacity building and information exchange

27. Efforts should be made to improve the capability of regulatory authorities, particularly those of developing countries, to assess, manage and communicate risks, including enforcement, associated with foods derived from modern biotechnology or to interpret assessments undertaken by other authorities or recognised expert bodies, including access to analytical technology. In addition capacity building for developing countries either through bilateral arrangements or with assistance of international organizations should be directed toward effective application of these principles[10].

28. Regulatory authorities, international organisations and expert bodies and industry should facilitate through appropriate contact points including but not limited to Codex Contact Points and other appropriate means, the exchange of information including the information on analytical methods.

Rewiew processes

29. Risk analysis methodology and its application should be consistent with new scientific knowledge and other information relevant to risk analysis.

30. Recognizing the rapid pace of development in the field of biotechnology, the approach to safety assessments of foods derived from modern biotechnology should be reviewed when necessary to ensure that emerging scientific information is incorporated into the risk analysis. When new scientific information relevant to a risk assessment becomes available the assessment should be reviewed to incorporate that information and, if necessary, risk management measures adapted accordingly ●

10 Reference is made to technical assistance of provisions in Article 9 of the SPS Agreement and Article 11 of the TBT Agreement.

Appendix 2.
Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants CAC/GL 45-2003

Section 1 – Scope

1. This Guideline supports the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology. It addresses safety and nutritional aspects of foods consisting of, or derived from, plants that have a history of safe use as sources of food, and that have been modified by modern biotechnology to exhibit new or altered expression of traits.

2. This document does not address animal feed or animals fed with the feed. This document also does not address environmental risks.

3. The Codex principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities such as food additives and pesticide residues, or a specific chemical or microbial contaminant that have identifiable hazards and risks; they are not intended to apply to whole foods as such. Indeed, few foods have been assessed scientifically in a manner that would fully characterise all risks associated with the food. Further, many foods contain substances that would likely be found harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. This approach is based on the principle that the safety of foods derived from new plant varieties, including recombinant-DNA plants, is assessed relative to the conventional counterpart having a history of safe use, taking into account both intended and unintended effects. Rather than trying to identify every hazard associated with a particular food, the intention is to identify new or altered hazards relative to the conventional counterpart.

5. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology. If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and if necessary further risk assessment, the food would be subjected to risk management considerations in accordance with the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology before it is considered for commercial distribution.

6. Risk management measures such as post-market monitoring of consumer health effects may assist the risk assessment process. These are discussed in paragraph 20 of the Principles for the Risk Analysis of Foods derived from Modern Biotechnology.

7. The Guideline describes the recommended approach to making safety assessments of foods derived from recombinant-DNA plants where a conventional counterpart exists, and identifies the data and information that are generally applicable to making such assessments. While this Guideline is designed for foods derived from recombinant- DNA plants, the approach described could, in general, be applied to foods derived from plants that have been altered by other techniques.

Section 2 – Definition

8. The definitions below apply to this Guideline:

- "Recombinant-DNA Plant"– means a plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.
- "Conventional Counterpart" – means a related plant variety, its components and/or products for which there is experience of establishing safety based on common use as food[1].

Section 3 – Introduction to food safety assessment

9. Traditionally, new varieties of food plants have not been systematically subjected to extensive chemical, toxicological, or nutritional evaluation prior to marketing, with the exception of foods for specific groups, such as infants, where the food may constitute a substantial portion of the diet. Thus, new varieties of corn, soya, potatoes and other common food plants are evaluated by breeders for agronomic and phenotypic characteristics, but generally, foods derived from such

[1] It is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

new plant varieties are not subjected to the rigorous and extensive food safety testing procedures, including studies in animals, that are typical of chemicals such as food additives or pesticide residues that may be present in food.

10. The use of animal models for assessing toxicological endpoints is a major element in the risk assessment of many compounds such as pesticides. In most cases, however, the substance to be tested is well characterised, of known purity, of no particular nutritional value, and, human exposure to it is generally low. It is therefore relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

11. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, often characterised by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects which are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

12. Due to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods derived from food plants, including recombinant- DNA plants. This has been addressed by the development of a multidisciplinary approach for assessing safety which takes into account both intended and unintended changes that may occur in the plant or in the foods derived from it, using the concept of substantial equivalence.

13. The concept of substantial equivalence is a key step in the safety assessment process. However, it is not a safety assessment in itself; rather it represents the starting point which is used to structure the safety assessment of a new food relative to its conventional counterpart. This concept is used to identify similarities and differences between the new food and its conventional counterpart[2]. It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods derived from recombinant-DNA plants. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the new product can be considered relative to its conventional counterpart.

Unintended effects

14. In achieving the objective of conferring a specific target trait (intended effect) to a plant by the insertion of defined DNA sequences, additional traits could, in some cases, be acquired or existing traits could be lost or modified (unintended effects). The potential occurrence of unintended effects is not restricted to the use of *in vitro* nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in conventional breeding. Unintended effects may be deleterious, beneficial, or neutral with respect to the health of the plant or the safety of foods derived from the plant. Unintended effects in recombinant-DNA plants may also arise through the insertion of DNA sequences and/or they may arise through subsequent conventional breeding of the recombinant-DNA plant. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA plant would have an unexpected, adverse effect on human health.

15. Unintended effects can result from the random insertion of DNA sequences into the plant genome which may cause disruption or silencing of existing genes, activation of silent genes, or modifications in the expression of existing genes. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels may give rise to secondary biochemical

[2] The concept of *substantial equivalence* as described in the report of the 2000 joint FAO /WHO expert consultations (Document WHO/SDE/PHE/FOS/00.6, WHO, Geneva, 2000).

effects or changes in the regulation of metabolic pathways and/or altered levels of metabolites.

16. Unintended effects due to genetic modification may be subdivided into two groups: those that are "predictable" and those that are “unexpected”. Many unintended effects are largely predictable based on knowledge of the inserted trait and its metabolic connections or of the site of insertion. Due to the expanding information on plant genome and the increased specificity in terms of genetic materials introduced through recombinant-DNA techniques compared with other forms of plant breeding, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse potential changes at the level of gene transcription and message translation that could lead to unintended effects.

17. The safety assessment of foods derived from recombinant-DNA plants involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information are necessary to assess unintended effects because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, provide assurance that the food is unlikely to have an adverse effect on human health. The assessment for unintended effects takes into account the agronomic/phenotypic characteristics of the plant that are typically observed by breeders in selecting new varieties for commercialization. These observations by breeders provide a first screen for plants that exhibit unintended traits. New varieties that pass this screen are subjected to safety assessment as described in Sections 4 and 5.

Framework of food safety assessment

18. The safety assessment of a food derived from a recombinant-DNA plant follows a stepwise process of addressing relevant factors that include:

A) Description of the recombinant-DNA plant;
B) Description of the host plant and its use as food;
C) Description of the donor organism(s);
D) Description of the genetic modification(s);
E) Characterization of the genetic modification(s);
F) Safety assessment:
  a) expressed substances (non-nucleic acid substances);
  b) compositional analyses of key components;
  c) evaluation of metabolites ;
  d) food processing;
  e) nutritional modification; and
G) Other considerations.

19. In certain cases, the characteristics of the product may necessitate development of additional data and information to address issues that are unique to the product under review.

20. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, Good Laboratory Practice. Primary data should be made available to regulatory authorities at request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

21. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. The expected endpoint of such an assessment will be a conclusion regarding whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. In essence, therefore, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed and if so to make well-informed and appropriate decisions.

Section 4 – General consideration

Description of the recombinant-DNA plant

22. A description of the recombinant-DNA plant being presented for safety assessment should be provided. This description should identify the crop, the transformation event(s) to be reviewed and the type and purpose of the modification. This description should be sufficient to aid in understanding the nature of the food being submitted for safety assessment.

Description of the host plant and its use as food

23. A comprehensive description of the host plant should be provided. The necessary data and information

should include, but need not be restricted to:

A) common or usual name; scientific name; and, taxonomic classification;

B) history of cultivation and development through breeding, in particular identifying traits that may adversely impact on human health ;

C) information on the host plant's genotype and phenotype relevant to its safety, including any known toxicity or allergenicity; and

D) history of safe use for consumption as food.

24. Relevant phenotypic information should be provided not only for the host plant, but also for related species and for plants that have made or may make a significant contribution to the genetic background of the host plant.

25. The history of use may include information on how the plant is typically cultivated, transported and stored, whether special processing is required to make the plant safe to eat, and the plant's normal role in the diet (*e.g.* which part of the plant is used as a food source, whether its consumption is important in particular subgroups of the population, what important macro- or micro-nutrients it contributes to the diet).

Description of the donor organism(s)

26. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health (*e.g.* presence of anti-nutrients). The description of the donor organism(s) should include:

A) its usual or common name;

B) scientific name;

C) taxonomic classification;

D) information about the natural history as concerns food safety;

E) information on naturally occurring toxins, anti-nutrients and allergens; for microorganisms, additional information on pathogenicity and the relationship to known pathogens; and

F) information on the past and present use, if any, in the food supply and exposure route(s) other than intendedfood use (*e.g.* possible presence as contaminants).

Description of the genetic modification(s)

27. Sufficient information should be provided on the genetic modification to allow for the identification of all genetic material potentially delivered to the host plant and to provide the necessary information for the analysis of the data supporting the characterization of the DNA inserted in the plant.

28. The description of the transformation process should include:

A) information on the specific method used for the transformation (*e.g.*Agrobacterium-mediated transformation);

B) information, if applicable, on the DNA used to modify the plant (*e.g.* helper plasmids), including the source (*e.g.* plant, microbial, viral, synthetic), identity and expected function in the plant; and

C) intermediate host organisms including the organisms (*e.g.* bacteria) used to produce or process DNA for transformation of the host organism.

29. Information should be provided on the DNA to be introduced, including:

A) the characterization of all the genetic components including marker genes, regulatory and other elements affecting the function of the DNA;

B) the size and identity;

C) the location and orientation of the sequence in the final vector/construct; and

D) the function.

Characterization of the genetic modification(s)

30. In order to provide clear understanding of the impact on the composition and safety of foods derived from recombinant-DNA plants, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out.

31. Information should be provided on the DNA insertions into the plant genome; this should include:

A) the characterization and description of the inserted genetic materials;

B) the number of insertion sites;

C) the organisation of the inserted genetic material at each insertion site including copy number and sequence data of the inserted material and of the surrounding region, sufficient to identify any substances expressed as a consequence of the inserted material, or, where more appropriate, other information such as analysis of transcripts or

expression products to identify any new substances that may be present in the food; and

D) identification of any open reading frames within the inserted DNA or created by the insertions with contiguous plant genomic DNA including those that could result in fusion proteins.

32. Information should be provided on any expressed substances in the recombinant-DNA plant; this should include:

A) the gene product(s) (*e.g.* a protein or an untranslated RNA);

B) the gene product(s)' function;

C) the phenotypic description of the new trait(s);

D) the level and site of expression in the plant of the expressed gene product(s), and the levels of its metabolites in the plant, particularly in the edible portions; and

E) where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous mRNA or protein.

33. In addition, information should be provided:

A) to demonstrate whether the arrangement of the genetic material used for insertion has been conserved or whether significant rearrangements have occurred upon integration;

B) to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;

C) to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable through several generations consistent with laws of inheritance. It may be necessary to examine the inheritance of the DNA insert itself or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly;

D) to demonstrate whether the newly expressed trait(s) are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene;

E) to indicate whether there is any evidence to suggest that one or several genes in the host plant has been affected by the transformation process; and

F) to confirm the identity and expression pattern of any new fusion proteins.

Safety assessment

Expressed Substances (non-nucleic acid substances)

Assessment of possible toxicity

34. *In vitro* nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in plants. The new substances can be conventional components of plant foods such as proteins, fats, carbohydrates or vitamins which are novel in the context of that recombinant-DNA plant. New substances might also include new metabolites resulting from the activity of enzymes generated by the expression of the introduced DNA.

35. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations and mean values. Current dietary exposure and possible effects on population sub-groups should also be considered.

36. Information should be provided to ensure that genes coding for known toxins or anti-nutrients present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic or anti-nutritious characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, since conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate anti-nutrients or toxicants.

37. For the reasons described in Section 3, conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary.

38. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (*e.g.* protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[3] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been

[3] Guidelines for oral toxicity studies have been developed in international fora, for example, the OECD Guidelines for the Testing of Chemicals.

consumed safely in food, and taking into account its biological function in the plant where known.

39. Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the plant of the substance and dietary exposure. The type of studies to be performed may include studies on metabolism, toxicokinetics, sub-chronic toxicity, chronic toxicity/carcinogenicity, reproduction and development toxicity according to the traditional toxicological approach.

40. This may require the isolation of the new substance from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case, the material should be shown to be biochemically, structurally, and functionally equivalent to that produced in the recombinant-DNA plant.

Assessment of possible allergenicity (proteins)

41. When the protein(s) resulting from the inserted gene is present in the food, it should be assessed for potential allergenicity in all cases. An integrated, stepwise, case-by-case approach used in the assessment of the potential allergenicity of the newly-expressed protein(s) should rely upon various criteria used in combination (since no single criterion is sufficiently predictive on either allergenicity or non-allergenicity). As noted in paragraph 20, the data should be obtained using sound scientific methods. A detailed presentation of issues to be considered can be found in the Annex to this document[4].

42. The newly expressed proteins in foods derived from recombinant-DNA plants should be evaluated for any possible role in the elicitation of gluten-sensitive enteropathy, if the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains.

43. The transfer of genes from commonly allergenic foods and from foods known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

[4] The FAO/WHO expert consultation 2001 report, which includes reference to several decision trees, was used in developing the Annex to these guidelines.

Compositional Analyses of Key Components

44. Analyses of concentrations of key components[5] of the recombinant-DNA plant and, especially those typical of the food, should be compared with an equivalent analysis of a conventional counterpart grown and harvested under the same conditions. In some cases, a further comparison with the recombinant-DNA plant grown under its expected agronomic conditions may need to be considered (*e.g.* application of an herbicide). The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. The comparator(s) used in this assessment should ideally be the near isogenic parental line.

In practice, this may not be feasible at all times, in which case a line as close as possible should be chosen. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that are nutritionally important or that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

45. The location of trial sites should be representative of the range of environmental conditions under which the plant varieties would be expected to be grown. The number of trial sites should be sufficient to allow accurate assessment of compositional characteristics over this range. Similarly, trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature. To minimise environmental effects, and to reduce any effect from naturally occurring genotypic variation within a crop variety, each trial site should be replicated. An adequate number of plants should be sampled and the methods of analysis should be sufficiently sensitive and specific to detect variations in key components.

Evaluation of Metabolites

46. Some recombinant-DNA plants may have been modified in a manner that could result in new or altered levels of various metabolites in the food. Consideration should be given to the potential for the accumulation of metabolites in the food that would adversely affect

[5] Key nutrients or key anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates as nutrients or enzyme inhibitors as anti-nutrients) or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased, selenium in wheat) and allergens.

human health. Safety assessment of such plants requires investigation of residue and metabolite levels in the food and assessment of any alterations in nutrient profile. Where altered residue or metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (*e.g.* procedures for assessing the human safety of chemicals in foods).

Food Processing

47. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.

Nutritional Modification

48. The assessment of possible compositional changes to key nutrients, which should be conducted for all recombinant- DNA plants, has already been addressed under 'Compositional analyses of key components'. However, foods derived from recombinant-DNA plants that have undergone modification to intentionally alter nutritional quality or functionality should be subjected to additional nutritional assessment to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

49. Information about the known patterns of use and consumption of a food, and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA plant. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.

50. The use of plant breeding, including *in vitro* nucleic acid techniques, to change nutrient levels in crops can result in broad changes to the nutrient profile in two ways. The intended modification in plant constituents could change the overall nutrient profile of the plant product and this change could affect the nutritional status of individuals consuming the food. Unexpected alterations in nutrients could have the same effect. Although the recombinant-DNA plant components may be individually assessed as safe, the impact of the change on the overall nutrient profile should be determined.

51. When the modification results in a food product, such as vegetable oil, with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods or food components whose nutritional composition is closer to that of the food derived from recombinant-DNA plant) as appropriate comparators to assess the nutritional impact of the food.

52. Because of geographical and cultural variation in food consumption patterns, nutritional changes to a specific food may have a greater impact in some geographical areas or in some cultural population than in others. Some food plants serve as the major source of a particular nutrient in some populations. The nutrient and the populations affected should be identified.

53. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.

Section 5 – Other considerations

Potential accumulation of substances significant to human health

54. Some recombinant-DNA plants may exhibit traits (*e.g.* herbicide tolerance) which may indirectly

result in the potential for accumulation of pesticide residues, altered metabolites of such residues, toxic metabolites, contaminants, or other substances which may be relevant to human health. The safety assessment should take this potential for accumulation into account. Conventional procedures for establishing the safety of such compounds (*e.g.* procedures for assessing the human safety of chemicals) should be applied.

Use of antibiotic resistance marker genes

55. Alternative transformation technologies that do not result in antibiotic resistance marker genes in foods should be used in the future development of recombinant-DNA plants, where such technologies are available and demonstrated to be safe.

56. Gene transfer from plants and their food products to gut microorganisms or human cells is considered a rare possibility because of the many complex and unlikely events that would need to occur consecutively. Nevertheless, the possibility of such events cannot be completely discounted[6].

57. In assessing safety of foods containing antibiotic resistance marker genes, the following factors should be considered:

A) the clinical and veterinary use and importance of the antibiotic in question;
   (Certain antibiotics are the only drug available to treat some clinical conditions (*e.g.* vancomycin for use in treating certain staphylococcal infections). Marker genes encoding resistance to such antibiotics should not be used in recombinant-DNA plants.)
B) whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficacy of the orally administered antibiotic; and
   (This assessment should provide an estimate of the amount of orally ingested antibiotic that could be degraded by the presence of the enzyme in food, taking into account factors such as dosage of the antibiotic, amount of enzyme likely to remain in food following exposure to digestive conditions, including neutral or alkaline stomach conditions and the need for enzyme cofactors (*e.g.* ATP) for enzymatic activity and estimated concentration of such factors in food.)
C) safety of the gene product, as would be the case for any other expressed gene product.

6 In cases where there are high levels of naturally occurring bacteria which are resistant to the antibiotic, the likelihood of such bacteria transferring this resistance to other bacteria will be orders of magnitude higher than the likelihood of transfer between ingested foods and bacteria.

58. If evaluation of the data and information suggests that the presence of the antibiotic resistance marker gene or gene product presents risks to human health, the marker gene or gene product should not be present in the food. Antibiotic resistance genes used in food production that encode resistance to clinically used antibiotics should not be present in foods.

Review of safety assessments

59. The goal of the safety assessment is a conclusion as to whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

Annex 1. Assessment of possible allergenicity

Section 1 – Introduction

1. All newly expressed proteins[7] in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

3. The endpoint of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

7 This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing glutensensitive or other enteropathies. The issue of enteropathies is already addressed in Assessment of possible allergenicity(proteins), paragraph 42 of the Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant- DNA Plants. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

Section 2 – Assessment strategy

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or, acid and enzymatic treatment.

5. As there is no single test that can predict the likely human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA plant. Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (*i.e.*: eukaryotic *vs.* prokaryotic systems) may have an impact on the allergenic potential of the protein.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

Section 3 – Initial assessment

Section 3.1 – Source of the protein

7. As part of the data supporting the safety of foods derived from recombinant-DNA plants, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available.

Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

Section 3.2 – Amino acid sequence homology

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results[8]. Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known

8 It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

Section 3.3 – Pepsin resistance

12. Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential[9]. Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided[10].

Section 4 – Specific serum screening

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals[11]. In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols[12]. A positive result in such tests would indicate a potential allergen.

Section 5 – Other considerations

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute toward an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing which would be applied and its effects on the presence of the protein in the final food product.

17. As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

9 The method outlined in the U.S. Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood et al. 1996).

10 Report of Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (2001): Section "6.4 Pepsin Resistance".

11 According to the Joint Report of the FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (22-25 January 2001, Rome, Italy) a minimum of 8 relevant sera is required to achieve a 99% certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

12 Ex vivo procedure is described as the testing for allergenicity using cells or tissue culture from allergic human subjects (Report of Joint FAO/WHO Expert Consultation on Allergenicity of Foods derived from Biotechnology).

Annex 2. Food safety assessment of foods derived from recombinant-dna plants modified for nutritional or health benefits

Section 1 – Introduction

1. General guidance for the safety assessment of foods derived from recombinant-DNA plants is provided in the Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (CAC/GL 45-2003) (Codex Plant Guideline). This Annex provides additional considerations that are specific to foods modified for nutritional or health benefits. The document does not extend beyond a safety assessment and therefore, it does not cover assessment of the benefits themselves or any corresponding health claims, or risk-management[13] measures.

2. The following factors determine whether a recombinant-DNA plant is a recombinant-DNA Plant Modified for Nutritional or Health Benefits, and as such within the scope of this Annex:

a) the recombinant-DNA plant exhibits a particular trait in portion(s) of the plant intended for food use, and;
b) The trait is a result of i) introduction of a new nutrient(s) or related substance(s), or ii) alteration of either the quantity or bioavailability of a nutrient(s) or related substance(s), iii) removal or reduction of undesirable substance(s) (e.g. allergens or toxicants), or iv) alteration of the interaction(s) of nutritional or health relevance of these substances.

Section 2 – Definition

3. The definition below applies to this Annex:

Nutrient[14] – means any substance normally consumed as a constituent of food:

a) which provides energy; or
b) which is needed for growth and development and maintenance of healthy life; or
c) a deficit of which will cause characteristic biochemical or physiological changes to occur.

4. This Annex draws, where appropriate, on the definitions of key nutritional concepts to be found or to be developed in relevant Codex texts, especially those elaborated by the Codex Committee on Nutrition and Foods for Special Dietary Uses.

13 Principles for the Risk Analysis of Foods Derived from Modern Biotechnology (CAC/GL 44-2003, paragraph 19).

14 General Principles for the Addition of Essential Nutrients to Foods (CAC/GL 09-1987).

Section 3 – Food safety assessment

5. The Codex General Principles for the Addition of Essential Nutrients to Foods (CAC/GL 09-1987) are generally applicable to the assessment of food derived from a plant which is modified by increasing the amount of a nutrient(s) or related substance(s) available for absorption and metabolism. The Food Safety Framework outlined within the Codex Plant Guideline[15] applies to the overall safety assessment of a food derived from a recombinant-DNA plant modified for nutritional or health benefits. This Annex presents additional considerations regarding the food safety assessment of those foods.

6. Foods derived from recombinant-DNA plants modified for nutritional or health benefits may benefit certain populations/sub populations, while other populations/sub populations may be at risk from the same food[16].

7. Rather than trying to identify every hazard associated with a particular food, the intention of a safety assessment of food derived from recombinant-DNA plants is the identification of new or altered hazards relative to the conventional counterpart[17]. Since recombinant-DNA plants modified for nutritional or health benefits result in food products with a composition that may be significantly different from their conventional counterparts, the choice of an appropriate comparator[18] is of great importance for the safety assessment addressed in this Annex. Those alterations identified in a plant modified to obtain nutritional or health benefits are the subject of this safety assessment.

8. Upper levels of intake for many nutrients that have been set out by some national, regional and international bodies[19] may be considered, as appropriate. The basis for their derivation should also be considered in order to assess the public health implications of exceeding these levels.

9. The safety assessment of related substances should follow a case-by-case approach taking into account upper levels as well as other values, where appropriate.

15 Paragraphs 18-21 (Safety Framework) and 48-53 (Nutrition Modification).

16 Further guidance for susceptible and high-risk population groups is provided in paragraph 49 of the Codex Plant Guideline.

17 Codex Plant Guideline, paragraph 4 .

18 Codex Plant Guideline, paragraph 51.

19 Where such guidance is not provided by Codex, information provided by the FAO/WHO may be preferably considered.

10. Although it is preferable to use a scientifically-determined upper level of intake of a specific nutrient or related substance, when no such value has been determined, consideration may be given to an established history of safe use for nutrients or related substances that are consumed in the diet if the expected or foreseeable exposure would be consistent with those historical safe levels.

11. With conventional fortification of food, typically a nutrient or a related substance is added at controlled concentrations and its chemical form is characterized. Levels of plant nutrients or related substances may vary in both conventionally bred and recombinant-DNA plants due to growing conditions. In addition, more than one chemical form of the nutrient might be expressed in the food as a result of the modification and these may not be characterized from a nutrition perspective. Where appropriate, information may be needed on the different chemical forms of the nutrient(s) or related substance(s) expressed in the portion of the plant intended for food use and their respective levels .

12. Bioavailability of the nutrient(s), related substance(s), or undesirable substance(s) in the food that were the subject of the modification in the recombinant-DNA plant should be established, where appropriate. If more than one chemical form of the nutrient(s) or related substance(s) is present, their combined bioavailability should be established, where appropriate.

13. Bioavailability will vary for different nutrients, and methods of testing for bioavailability should be relevant to the nutrient, and the food containing the nutrient, as well as the health, nutritional status and dietary practices of the specific populations consuming the food. *In vitro* and *in vivo* methods to determine bioavailability exist, the latter conducted in animals and in humans. *In vitro* methods can provide information to assess extent of release of a substance from plant tissues during the digestive process. *In vivo* studies in animals are of limited value in assessing nutritional value or nutrient bioavailability for humans and would require careful design in order to be relevant. In vivo studies, in particular, human studies may provide more relevant information about whether and to what extent the nutrient or related substance is bioavailable.

14. Guidance on dietary exposure assessment of foods derived from recombinant-DNA plants with nutritional modifications is provided in paragraph 49 of the Codex Plant Guideline. In the context of this Annex, dietary exposure assessment is the estimation of the concentration of the nutrient(s) or related substance(s) in a food, the expected or foreseeable consumption of that food, and any known factors that influence bioavailability. Exposure to a nutrient(s) or related substance(s) should be evaluated in the context of the total diet and the assessment should be carried out based on the customary dietary consumption, by the relevant population(s), of the corresponding food that is likely to be displaced. When evaluating the exposure, it is appropriate to consider information on whether the consumption of the modified food could lead to adverse nutritional effects as compared to consumption of the food that it is intended to replace. Most, if not all, aspects of exposure assessment are not unique to recombinant-DNA plants modified for nutritional or health benefits[20].

15. The first step of an exposure assessment is determining the level(s) of the substance(s) in question in the portion of the plant intended for food use. Guidance on determining changes in levels of these substances is provided in the Codex Plant Guideline.[21]

16. Consumption patterns will vary from country to country depending on the importance of the food in the diet(s) of a given population(s). Therefore, it is recommended that consumption estimates are based on national or regional food consumption data when available, using existing guidance on estimation of exposure in a given population(s)[22]. When national or regional food consumption data is unavailable, food availability data may provide a useful resource[23].

17. To assess the safety of a food derived from a recombinant-DNA plant modified for a nutritional or health benefit, the estimated intake of the nutrient or related substance in the population(s) is compared with the nutritional or toxicological reference values, such as upper levels of intake, ADIs for that nutrient or related substance, where these values exist. This may involve assessments of different consumption scenarios against the relevant nutritional reference value, taking into account possible changes in bioavailability, or extend to probabilistic methods that characterise the distribution of exposures within the relevant population(s).

20 Additional applicable guidance on dietary exposure assessment of nutrients and related substances is provided in the Report of a Joint FAO/WHO Technical Workshop on Nutrient Risk Assessment. WHO Headquarters, Geneva, Switzerland, 2-6 May 2005.

21 Paragraphs 44 and 45.

22 A Model for Establishing Upper Levels of Intake for Nutrients and Related Substances. Report of a Joint FAO/WHO Technical Workshop on Nutrient Risk Assessment. WHO Headquarters, Geneva, Switzerland, 2-6 May 2005.

23 Data on staple food products may also be supplemented by information from FAO Food Balance Sheets.

Annex 3. Food safety assessment in situations of low-level presence of recombinant-dna plant material in food

Section 1 – Preamble

1. An increasing number of recombinant–DNA plants are being authorized for commercialization. However, they are authorized at different rates in different countries. As a consequence of these asymmetric authorizations, low levels of recombinant DNA plant materials that have passed a food safety assessment according to the Codex Guideline for the conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (CAC/GL 45-2003) (Codex Plant Guideline) in one or more countries may on occasion be present in food in importing countries in which the food safety of the relevant recombinant-DNA plants has not been determined.

2. This Annex describes the recommended approach to the food safety assessment in such situations of low-level presence of recombinant-DNA plant material or in advance preparation for such potential circumstances[24].

3. This Annex also describes data and information sharing mechanisms to facilitate utilization of the Annex and to determine whether it should apply.

4. This Annex can be applied in two different dietary exposure situations:

a) That involving commodities, such as grains, beans or oil seeds, in which exposure to food from a variety not authorized in the importing country would likely be to dilute low level amounts at any one time. This would likely be the more common situation of low-level presence of recombinant-DNA plant material. Because any food serving of grains, beans or oil seeds would almost necessarily come from multiple plants, and because of how these types of commodities generally are sourced from multiple farms, are commingled in grain elevators, are further commingled in export shipments, at import and when used in processed foods, any inadvertently commingled material derived from recombinant-DNA plant varieties would be present only at a low level in any individual serving of food.

b) That involving foods that are commonly consumed whole and undiluted, such as some fruits and vegetables like potatoes, tomatoes, and papaya, in which exposure would be rare but could be to an undiluted form of the unauthorized recombinant-DNA plant material. While the likelihood of consuming material from such an unauthorized variety would be low and the likelihood of repeated consumption would be much lower, any such consumption might be of an entire unauthorized fruit or vegetable.

5. In both cases, the dietary exposure will be significantly lower than would be considered in a food safety assessment of the recombinant-DNA plant according to the Codex Plant Guideline. As a result, only certain elements of the Codex Plant Guideline will be relevant and therefore are included in this Annex.

6. This Annex does not:

- address risk management measures; national authorities will determine when a recombinant-DNA plant material is present at a level low enough for this Annex to be appropriate;
- preclude national authorities from conducting a safety assessment according to the Codex Plant Guideline; countries can decide when and how to use the Annex within the context of their regulatory systems; or
- eliminate the responsibility of industries, exporters and, when applicable, national competent authorities to continue to meet countries' relevant import requirements, including in relation to unauthorized recombinant-DNA plant material.

Section 2 – General and other considerations

7. For the food safety assessment in situations of low-level presence of recombinant DNA plant materials in food, sections 4 and 5 of the Codex Plant Guideline apply as amended as follows. The applicable paragraphs are specifically indicated. Those paragraphs of the Codex Plant Guidelines that are not listed can be omitted from consideration.

Description of the recombinant-dna plant

8. Paragraph 22 of the Codex Plant Guideline applies.

Description of the host plant and its use as a food

9. Paragraphs 23, 24 and 25 of the Codex Plant Guideline apply.

[24] This guidance is not intended for a recombinant-DNA plant that was not authorized in an importing country as a result of that country's food safety assessment.

Description of the donor organism(s)

10. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. The description of the donor organism(s) should include:

A. its usual or common name;
B. scientific name;
C. taxonomic classification;
D. information about the natural history as concerns food safety;
E. information on naturally occurring toxins and allergens; for microorganisms, additional information on pathogenicity and the relationship to known pathogens; and,
F. information on past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g., possible presence as contaminants)[25].

Description of the genetic modification(s)

11. Paragraphs 27, 28 and 29 of the Codex Plant Guideline apply.

Characterization of the genetic modification(s)

12. Paragraphs 30 and 31 of the Codex Plant Guideline apply.

13. Information should be provided on any expressed substances in the recombinant-DNA plant; this should include: A) the gene product(s) (e.g. a protein or an untranslated RNA); B) the gene product(s)' function; C) the phenotypic description of the new trait(s); D) the level and site of expression in the plant of the expressed gene product(s), and the levels of its metabolites in the edible portions of the plant; and E) where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous mRNA or protein.[26]

14. Paragraph 33 of the Codex Plant Guideline applies.

Safety Assessment

Expressed Substances (non-nucleic acid substances)

Assessment of possible toxicity

15. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations and mean values.[27]

16. Information should be provided to ensure that genes coding for known toxins present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, since conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate toxicants.[28]

17. Paragraph 37 of the Codex Plant Guideline applies.

18. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. appropriate oral toxicity studies[29] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, and taking into account its biological function in the plant where known.[30]

19. Paragraphs 39 and 40 of the Codex Plant Guideline apply.

Assessment of possible allergenicity (proteins)

20. Paragraphs 41, 42 and 43 of the Codex Plant Guideline apply.

25 The text of this paragraph was adapted from paragraph 26 of the Codex Plant Guideline.
26 The text of this paragraph was adapted from paragraph 32 of the Codex Plant Guideline.
27 The text of this paragraph was adapted from paragraph 35 of the Codex Plant Guideline.
28 The text of this paragraph was adapted from paragraph 36 of the Codex Plant Guideline.
29 Guidelines for oral toxicity studies have been developed in international fora, for example, the OECD Guidelines for the Testing of Chemicals.
30 The text of this paragraph was adapted from paragraph 38 of the Codex Plant Guideline.

Analyses of Key Toxicants and Allergens

21. Analyses of key toxicants[31] and allergens are important in certain cases of foods from recombinant-DNA plants (e.g., those that are commonly consumed whole and undiluted, such as potatoes, tomatoes, and papaya). Analyses of concentrations of key toxicants and allergens of the recombinant-DNA plant typical of the food should be compared with an equivalent analysis of a conventional counterpart grown and harvested under the same conditions. The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. The comparator(s) used in this assessment should ideally be the near isogenic parental line. In practice, this may not be feasible at all times, in which case a line as close as possible should be chosen. The purpose of this comparison is to establish that substances that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.[32]

22. The location of trial sites should be representative of the range of environmental conditions under which the plant varieties would be expected to be grown. The number of trial sites should be sufficient to allow accurate assessment of key toxicants and allergens over this range. Similarly, trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature. To minimize environmental effects, and to reduce any effect from naturally occurring genotypic variation within a crop variety, each trial site should be replicated. An adequate number of plants should be sampled and the methods of analysis should be sufficiently sensitive and specific to detect variations in key toxicants and allergens.[33]

Evaluation of Metabolites

23. Some recombinant-DNA plants may have been modified in a manner that could result in new or altered levels of various metabolites in the food. In certain cases of foods from recombinant-DNA plants (e.g., those that are commonly consumed whole and undiluted), consideration should be given to the potential for the accumulation of metabolites in the food that would adversely affect human health. Food safety assessment in situations of low level presence of recombinant-DNA material in foods from such plants requires investigation of residue and metabolite levels in the food. Where altered residue or metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g. procedures for assessing the human safety of chemicals in foods).[34]

Food Processing

24. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.[35]

Potential accumulation of substances significant to human health

25. Some recombinant-DNA plants may exhibit traits (e.g. herbicide tolerance) which may indirectly result in the potential for accumulation of pesticide residues, altered metabolites of such residues, toxic metabolites, contaminants, or other substances which may be relevant to human health. In certain cases of foods from recombinant-DNA plants (e.g. those that are commonly consumed whole and undiluted), the risk assessment should take this potential for accumulation into account. Conventional procedures for establishing the safety of such compounds (e.g. procedures for assessing the human safety of chemicals) should be applied.[36]

Use of antibiotic resistance marker genes

26. Paragraphs 55, 56, 57 and 58 of the Codex Plant Guideline apply.

31 Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased).

32 The text of this paragraph was adapted from paragraph 44 of the Codex Plant Guideline.

33 The text of this paragraph was adapted from paragraph 45 of the Codex Plant Guideline.

34 The text of this paragraph was adapted from paragraph 46 of the Codex Plant Guideline.

35 The text of this paragraph was adapted from paragraph 47 of the Codex Plant Guideline.

36 The text of this paragraph was adapted from paragraph 54 of the Codex Plant Guideline.

Section 3 – Guidance on data and information sharing

27. In order for Codex Members to use this Annex, it is essential that they have access to requisite data and information.

28. Codex Members should make available to a publicly accessible central database to be maintained by FAO information on recombinant-DNA plants authorized in accordance with the Codex Plant Guideline. This information should be presented in accordance with the following format:

a) name of product applicant;
b) summary of application;
c) country of authorization;
d) date of authorization;
e) scope of authorization;
f) unique identifier;
g) links to the information on the same product in other databases maintained by relevant international organizations, as appropriate;
h) summary of the safety assessment, which should be consistent with the framework of food safety assessment of the Codex Plant Guideline;
i)where detection method protocols and appropriate reference material (non-viable, or in certain circumstances, viable) suitable for low-level situation may be obtained[37]; and
j) contact details of the competent authority(s) responsible for the safety assessment and the product applicant.

29. This process should facilitate rapid access by importing Codex Members to additional information relevant to the assessment of food safety assessment in situations of low-level presence of recombinant-DNA plant material in foods in accordance with this Annex.

30. The authorizing Codex Members should make available complementary information to other Codex Members on its safety assessment in accordance with the Codex Plant Guideline, in conformity with its regulatory/legal framework.

31. The product applicant should provide further information and clarification as necessary to allow the assessment according to this Annex to proceed, as well as a validated protocol for an event-specific or trait-specific detection method suitable for low level situations and appropriate reference materials (non-viable, or in certain circumstances, viable). This is without prejudice to legitimate concerns to safeguard the confidentiality of commercial and industrial information.

32. As appropriate, new scientific information relevant to the conclusions of the food safety assessment conducted in accordance with the Codex Plant Guideline by the authorizing Codex member should be made available ●

34 This information may be provided by the product applicant or in some cases by Codex members.

Part Two

Tools and Techniques for trainers

12. Preparing and delivering a workshop

Workshop preparation

The success of a workshop typically reflects the effort that went into its preparation. Below are some of the key activities that workshop organizers may wish to consider during the preparatory period.

1. Confirm that the required financial resources will be provided and accessible when required. Obtain the necessary institutional and administrative support to manage the workshop, including realistic budgeting and cost-control measures to avoid corruption and written agreements on auditing and cost-control measures.
2. Identify the goal(s) of the workshop. These may take the form of a statement of purpose or objectives, an agenda and/or a discussion paper that sets out key issues, and references or resources with which the participants may wish to familiarize themselves in preparation for the workshop. The goals of the workshop may include:
   - Introduction of participants to the concepts and principles used to frame the pre-market safety assessment of foods derived from recombinant-DNA plants.
   - Introduction of participants to types of information and data that they may be tasked with evaluating as safety assessors, using case studies of products approved for human consumption in a number of countries.
   - Emphasizing the multidisciplinary nature of the safety assessment of foods derived from recombinant-DNA plants using practical, hands-on exercises designed to simulate the team effort required.
3. Determine how many people can be accommodated at the trainers' workshop. The number of people invited will partly determine the process that is most appropriate for the stated purpose. Safety assessment workshops are most successful when they are iterative and the

Form 12.1. Terms of reference for participant selection

Checklist for desired profile

- ❑ Experience as a regulator or scientist active in agricultural biotechnology or a discipline relevant to the safety assessment of GM foods. Examples include molecular biology, plant breeding, biochemistry, immunology, toxicology, and human or livestock nutrition.
- ❑ Experience working in a multidisciplinary environment with people of different nationalities, ethnicity and cultural backgrounds.
- ❑ Familiarity with use of computers, on-line information communication and information retrieval.
- ❑ Experience of both public and private sector research and development.
- ❑ Publication record in both the scientific literature and more general interest press.
- ❑ Communication and presentation skills, particularly to different audiences.
- ❑ Advanced university degree in biological/agricultural sciences or an equivalent combination of education and experience.
- ❑ Excellent spoken/written [language]

participants are given group activities or exercises to carry out, rather than being passive recipients of information through a more typical seminar or lecture format. To maximize the effectiveness of such training it is suggested that the number of participants should be limited to approximately 20.

4. Participant selection is key to the success of a GM food safety assessment workshop and therefore it is very important to identify carefully those best to invite with reference to the workshop objectives. It may be possible to invite individual regulators/scientists to the workshop directly, but often, because of protocol considerations, letters of invitation must be sent to a director or senior administrator of a specific institution inviting them to select one or more delegates to participate in the workshop. In order to assist this person in selecting the appropriate participant(s), it is helpful to provide terms of reference that clearly describe the education and professional expertise that is required. A template for this is provided in Form 12.1.
5. Determine the appropriate way to invite participants to the workshop, whether in writing or by telephone, directly or via someone more senior in their organization. This will probably be determined by such factors as:
   a. decisions the workshop organizers make regarding the focus of the workshop;
   b. the organizational level from which the organizers wish to attract participants;
   c. the relationship the organizers may or may not have established already with potential participants;
   d. the extent to which invitees themselves are in a position to decide whether or not to attend.
6. Allow sufficient time between receipt of an invitation and the actual event to avoid conflicts with other previously scheduled commitments. Time is also required to make appropriate travel arrangements, including applications for entry visas, which for some countries can take several months.
7. Provide any relevant background material for review in advance. This might include:
   - Codex Principles for the Risk Analysis of Foods Derived from Modern Biotechnology (Appendix 1);
   - Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (Appendix 2);
   - selected case studies;
   - key references, especially those pertinent to the country/region in which the workshop is being given.
8. Identify a suitable location and venue for the workshop. If participants must travel to the location, the venue should be in close proximity to adequate lodging. Other issues to consider when selecting a venue are listed below.
   a. If small-group work is an important part of the agenda, the venue should have sufficient separate rooms.
   b. The group-work rooms should all have flip-chart stands, paper and markers to facilitate the small-group work/discussions.
   c. The plenary room must have the necessary "hardware" to accommodate the presenters. Typically this includes an LCD-type projector or overhead projector and a large screen.
   d. Depending on the size of the group, microphones for speakers and mobile "floor microphones" may be necessary to allow participants to ask questions and hear answers.
   e. Ideally, the seating arrangement in the plenary room should have all participants sitting around smaller tables for interaction during and after presentations. This is particularly important if break-out rooms are not available and small-group work must take place in the plenary room.

The following is a sample checklist that organizers may wish to use and/or adapt as they prepare for a workshop.

Form 12.2. Workshop preparation checklist

- ❑ Identify appropriate funding sources for the workshop and prepare written agreements on the objectives, size and structure of the workshop. All financial and administrative matters must be clarified in writing, including how the financial resources will be managed.
- ❑ Ensure that proper auditing will be performed and specify exactly what type of expenses will be covered by the workshop budget. Most often, a full-time position is required to take care of all practical matters starting at least 2 months prior to the workshop.
- ❑ Conduct pre-workshop meetings during which you will:
  - ❍ identify key collaborators and their contributions (host institute, sponsors, etc.);
  - ❍ identify facilitator(s);
  - ❍ generate a list of potential participants;
  - ❍ select a date for conducting the workshop;
  - ❍ decide on a list of potential speakers for each session for which a speaker is required.
- ❑ Estimate workshop costs and cost-sharing across partners if appropriate.
- ❑ Send invitations at least three weeks before the event for national workshops. For international workshops, 2 months' notice is the absolute minimum. If the travel costs will be covered by the organizers, clear and written instructions of the fare class, maximum costs and cost documentation must be provided.
- ❑ Identify and reserve a workshop location that (ideally) has:
  - ❍ a plenary room large enough for the number of participants;
  - ❍ break-out rooms, or a room large enough for small groups to work in the same room without disturbing each other;
  - ❍ on-site accommodation.
- ❑ Make arrangements with a food and beverage supplier for lunch and breaks. Take into account food needs/preferences for international workshops in which cultural and religious considerations may specify food choices.
- ❑ Finalize the agenda and confirm presenters, and clarify in writing their financial support (travel, accommodation, honorarium, etc.).
- ❑ Hold a training meeting for small-group facilitators 1 week prior to the workshop if small-group sessions are to be included.
- ❑ Collect necessary equipment and materials prior to the workshop:
  - ❍ notebooks and pens for participants;
  - ❍ pads of flip-chart paper and markers;
  - ❍ name tags;
  - ❍ copies of all handouts to be distributed.
- ❑ Obtain overhead and LCD projectors, including spare equipment, and ensure the availability of technical assistance prior to and during the workshop.

The workshop facilitator

A workshop facilitator acts as a guide for the entire workshop and as such should have excellent organizational and leadership skills. In the case of GM food safety assessment training workshops, the workshop organizer is often required to take on the role of facilitator as well. The following are some practical tips for facilitating workshops.

- Participants appreciate a meeting that starts and ends on time. Ensure that sufficient time is allotted for registration at the beginning of the workshop so that this activity can be completed before the workshop opens. Similarly, participant assessment or evaluation forms should be provided before the close of the workshop and enough time provided so that the attendees can complete these and hand them in before the workshop ends.
- Ensure that there is a registration table to assist participants as they arrive. Provide a sign-in sheet that lists name, organization, region of work and contact information for each person. Distribute name tags.
- Ensure that a list of participants is compiled and distributed by the end of the workshop. Consider asking participants to verify the accuracy of the information before they leave.
- Keep presenters and participants informed of the point they are at on the agenda. If the

Form 12.3. Sample agenda for 3-day workshop

GM Food Safety Assessment Workshop Agenda

Venue _____

Date _____

Day 1

8.00	Registration	
9.00	Opening, welcome	
	Workshop Introduction	
9.15	**Presentation 1:** Workshop overview	Presentation Module 1
9.30	Introduction of the participants and trainers	
	Part I: Concept of GM Food Safety Assessment and International Perspectives	
9.45	**Presentation 2:** Concepts and principles of GM food safety assessment, key international initiatives	Presentation Module 2 Ch. 2
10.30	*Coffee break*	
11.00	Introduction of three case studies • GM insect-resistant corn event MON 810 • GM high oleic acid soybeans • GM herbicide tolerant soybean	
12.00	Assigning working groups	
12.30	*Lunch*	
	Part II: Approach and Framework, Identification of Required Information	
13.30	**Presentation 3:** The comparative approach and the framework for the safety assessment of GM foods	Presentation Module 3 Ch. 3–4
14.30	**Working Group Session 1:** Using case studies, identify the description and review the sufficiency of the information on: • description of the recombinant-DNA plant • description of the host plant and its use as food • description of the donor organism(s) • description of the genetic modification(s)	
15.30	*Coffee break*	
16.00	**Plenary Session 1:** Report back and discussion for WG Session 1	
17.30	Summary and Conclusions of Day 1	

(Continued)

Form 12.3. (cont.)

Day 2

	Part III: Possible Toxicity & Allergenicity and Compositional Analysis	
9.00	**Presentation 4:** Characterization of the genetic modification(s), assessment of possible toxicity & allergenicity, and compositional analysis of key components	Presentation Module 4 Ch. 5–8
10.00	**Working Group Session 2:** Using case studies, evaluate possible toxicity	
10.30	*Coffee break*	
11.00	Working Group Session 2, cont.	
12.00	*Lunch*	
13.00	**Plenary Session 2:** Report back and discussion for WG Session 3	
14.00	**Working Group Session 3:** Using case studies, evaluate possible allergenicity	
15.30	*Coffee break*	
16.00	**Plenary Session 3:** Report back and discussion for WG Session 4	
17.30	Summary and Conclusions of Day 2	

Day 3

9.00	**Working Group Session 4:** Using case studies, identify and evaluate the compositional analysis	
10.30	*Coffee break*	
11.00	**Plenary Session 4:** Report back and discussion for WG Session 5 and overall WG sessions	
12.30	*Lunch*	
	Part IV: Risk Communication	
13.30	**Presentation 5:** Risk communication and safety assessment decisions	Presentation Module 5 Ch. 10
14.30	**Working Group Session 5:** Using case studies: • strategize the meaning of risk communication • prepare a decision document for the general public	
15.30	*Coffee break*	
16.00	**Plenary Session 5:** Report back and discussion for WG Session 6	
17.30	• Workshop evaluation • Handing out certificates • Concluding comments • Closing workshop	

workshop takes place over two or more days, announce the daily programme at the start of each day.

- Ensure that all presentation materials are available and ready to be used. In the case of PowerPoint or similar presentations, ensure that these are loaded and saved on a computer before the start of each day's presentations.
- Provide an overview of a particular segment of a workshop before asking participants to begin their small-group or workbook work.
- Move from group to group to get the flavour of discussions and to help clarify any questions participants may have. Depending on the number of workshop participants, it may be helpful also to involve others as small-group facilitators.
- Keep an eye on the clock and give participants advance notice of when they need to complete a segment of their work.
- Ensure that there is enough time allocated to discussion. Participants may often learn more from each other than from the lecturers!

The facilitator does not need to have all the answers; however, participants will look to the facilitator for guidance in exploring a question in order to arrive at an appropriate response. Facilitators must be prepared in advance for this. There should be a moderator for each session to facilitate and guide the discussion.

Workshop agenda

Creating an effective agenda is an important element of a productive workshop. The agenda communicates information regarding:

- topics of discussion;
- the presenter for each topic;
- the time allotted to each topic;
- the focus of the meeting.

Presentations and break-out sessions should be based on the safety assessment processes, including:

- description of the recombinant-DNA plant;
- description of the host plant and its use as food;
- description of the donor organisms(s);
- characterization of the genetic modification(s);
- safety assessment:
  - expressed substances (non-nucleic acid substances)
  - compositional analysis of key components
  - evaluation of metabolites
  - food processing
  - nutritional modification
  - other considerations.

A sample agenda is provided in the Form 12.3. Organizers may wish to use and/or adapt as they prepare for a workshop.

Workshop evaluation and certificates

Evaluation

It is important to get feedback on participants' experiences during the workshop and their plans for using the information. A workshop evaluation form should be distributed before the close of the workshop. A sample evaluation form is provided in the Form 12.4. Organizers may wish to use and/or adopt as they prepare for a workshop.

Box 12.1. Creating an effective agenda

1. Develop an agenda for your workshop that involves:
   - 20 participants with different levels of expertise;
   - lectures presenting the safety assessment principles and criteria based on the Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants;
   - group activities/exercises providing hands-on experience in the food safety assessment of these products using case studies.
2. Select appropriate case studies for your workshop.
   - Suitable for training?
     - Select a clear, simple example;
   - Feasible for the time frame of the workshop?
     - at least 15 minutes are needed for explanation of the case study at the beginning of the workshop
     - a break-out group discussion session usually takes at least 1 hour
     - a report-back session also takes at least 1 hour.

Box 12.2. Developing a workshop evaluation

1. Write down your key objectives for your workshop.
2. Establish measures (ratings, response options, open-ended questions) to assess whether the objectives have been achieved.
3. Develop questions using 1 and 2, noting the following:
   - an appropriate number of questions is 5 to 10;
   - always provide a space for comments;
   - consider providing 15 minutes to fill out and 5 minutes to collect the forms.

TIP: In order to ensure that everyone fills out an evaluation, organizers may consider awarding certificates of participation for workshop attendees. Participants receive their certificate once they have completed and handed in their evaluation form. It is important that the evaluation is done prior to the end of the programme.

Certificates

Certificates can be used as a positive reinforcement to certify that the trainee completed the workshop and to encourage active participation in the workshop.

The one-page certificate should contain brief information about the organizer (not FAO), date and duration of the course, location, course topics and the full name of the participant. The certificate should note that the person attended the training course and be signed and stamped as appropriate.

Workshop presentations

Visual aids

Visual aids should be clear, simple and necessary to the presentation. It is important to refer to slides/transparencies during the course of the presentation; the audience should be guided through the key points on each visual aid. Transparencies or slides that do not serve a legitimate purpose should be discarded. The use of too many visual aids may detract from the talk by distracting the audience from the presenter.

Training modules

The sample presentations may be useful for organizers/facilitators/trainers when preparing for a workshop (pages 86–102). A CD-ROM provides the modules in electronic format together with other relevant reference materials ●

Form 12.4. Sample Workshop Evaluation Form

Workshop Evaluation Form

Workshop name _____
Venue _____
Date _____

Your cooperation in completing this questionnaire is greatly appreciated. The information you provide will be useful in planning future events and will help the organizers and presenters to improve their materials and presentations.

Overall rating

In general, how would you rate this course?

- ☐ Excellent
- ☐ Good
- ☐ Average
- ☐ Fair
- ☐ Poor

Workshop objectives

The objectives of this workshop are listed below. Please indicate, on a scale of 1 to 5, if you believe these objectives have been achieved. A rating of 1 means the objective has not been achieved and 5 means the objective has been achieved fully.

Objective 1: [Type the objective here]
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5

Objective 2: [Type the objective here]
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5

Objective 3: [Type the objective here]
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5

Page 1

(Continued)

Form 12.4. (cont.)

Topics

In this section we would like you to rate the content, usefulness, supporting aids (e.g. slides, handouts, etc.) and time management of each presentation. When rating content, you should consider such factors as the rigor of the material (theory, soundness and methodology). With regard to usefulness, rate the topic in terms of its applicability/relevance to the workshop objectives. Factors to consider in assessing presentation include clarity, logical structure, good use of visual aids, etc. Please place a check in the box that most accurately represents your opinion of these factors.

	Content					*Usefulness*					*Presentation*					*Time management*				
Day 1	*Excellent*	*Good*	*Average*	*Fair*	*Poor*	*Excellent*	*Good*	*Average*	*Fair*	*Poor*	*Excellent*	*Good*	*Average*	*Fair*	*Poor*	*Excellent*	*Good*	*Average*	*Fair*	*Poor*
Presentation 1 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Presentation 2 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Presentation 3 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Activity 1 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Activity 2 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	Content					*Usefulness*					*Presentation*					*Time management*				
Day 2	*Excellent*	*Good*	*Average*	*Fair*	*Poor*	*Excellent*	*Good*	*Average*	*Fair*	*Poor*	*Excellent*	*Good*	*Average*	*Fair*	*Poor*	*Excellent*	*Good*	*Average*	*Fair*	*Poor*
Presentation 1 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Presentation 2 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Presentation 3 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Activity 1 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Activity 2 (type title)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

(Continued)

Form 12.4. (cont.)

Strengths and weaknesses

Please list what you consider to be three main **strengths** of the course.

1.

2.

3.

Please list what you consider to be three main **weaknesses** of the course.

1.

2.

3.

Page 3

(Continued)

Form 12.4. (cont.)

Additional topics

What additional topics should have been included in this course?
- and what topics should be excluded to allow this?

Logistical arrangements

Pre-meeting support	☐ Excellent	☐ Good	☐ Average	☐ Fair	☐ Poor
Accommodation	☐ Excellent	☐ Good	☐ Average	☐ Fair	☐ Poor
Meals	☐ Excellent	☐ Good	☐ Average	☐ Fair	☐ Poor
Organization and management	☐ Excellent	☐ Good	☐ Average	☐ Fair	☐ Poor

Other comments

Please write down any additional comments or suggestions you may have.

Page 4

Visual aids
Module 1
Workshop overview

GM food safety assessment

Presentation module 1

Workshop overview

Food and Agriculture Organization of the United Nations

GM food safety assessment

Workshop objectives

- **Assist in implementing internationally accepted** principles and guidelines
- Share many **countries' experiences of regulation and safety assessment methods**

Module 1 / slide 2

GM food safety assessment

Scope of the workshop

Train experts (training of trainers) to deliver workshops to

- **improve the capability of regulatory authorities**
- to **assess**, **manage** and **communicate** the potential risks associated with foods derived from recombinant-DNA plants

Module 1 / slide 3

GM food safety assessment

Presentations

- Concepts and principles of safety assessment
- Approach and frameworks of safety assessment
- Assessment of possible toxicity, possible allergenicity, and compositional analysis
- Risk communication and public policy issues

Module 1 / slide 4

GM food safety assessment

Working group sessions

- Break-out sessions with case studies will give participants hands-on exercises on preparing and conducting training workshops
- Case studies include:
  - MON 810 corn
  - GTS 40-3-2 soybeans
  - High oleic acid soybeans

Module 1 / slide 5

GM food safety assessment

Expected outcomes

Workshop provides:

- an overview of the international perspectives on safety assessment of foods derived from r-DNA plants (Codex and FAO/WHO)
- theoretical and practical experience in safety assessment methodology
- practical information on organizing and delivering training workshops

Properly trained regulators can:

- enhance the safety of foods, thereby not only improving the health of consumers but also ensuring the safety of foods entering international trade

Module 1 / slide 6

Visual aids
Module 1 (cont.)

GM food safety assessment

Agenda: day 1 morning

9.15	**Presentation 1.** Workshop overview
9.30	Introduction of the participants and trainers
9.45	**Presentation 2.** Concepts and principles of GM Food safety assessment, key international initiatives (Codex, FAO/WHO, OECD, etc.)
10.30	*Coffee break*
11.00	Introduction of three case studies • GM Insect Resistant Corn Event MON 810 • High Oleic Acid Soybeans • GM Herbicide Tolerant Soybeans
12.00	Assigning working groups
12.30	*Lunch*

Module 1 / slide 7

GM food safety assessment

Agenda: day 1 afternoon

13.30	**Presentation 3.** The comparative approach and the framework for the safety assessment of GM foods
14.30	**Working group session 1.** Using case studies, identify the description and review the sufficiency of the information on: • description of the recombinant-DNA plant • description of the host plant and its use as food • description of the donor organism(s) • description of the genetic modification(s)
15.30	*Coffee break*
16.00	**Plenary session 1.** Report back and discussion for WG Session 1
17.30	Summary and Conclusions of Day 1

Module 1 / slide 8

GM food safety assessment

Agenda: day 2

9.00	**Presentation 4.** Characterization of the genetic modification(s), assessment of possible toxicity & allergenicity, and compositional analysis of key components
10.00	**Working group session 2.** Evaluate possible toxicity
12.00	*Lunch*
13.00	**Plenary session 2.** Report back
14.00	**Working group session 3.** Possible allergenicity
15.30	*Coffee break*
16.00	**Plenary session 3.** Report back
17.30	Summary and Conclusions of Day 2

Module 1 / slide 9

GM food safety assessment

Agenda: day 3

9.00	**Working group session 4.** The compositional analysis
10.30	*Coffee break*
11.00	**Plenary session 4.** Report back
12.30	*Lunch*
13.30	**Presentation 5.** Risk communication
14.30	**Working group session 5.** Using case studies: strategize the meaning of risk communication; prepare a decision document for the general public
15.30	*Coffee break*
16.00	**Plenary session 5.** Report back
17.30	Workshop evaluation, certificates, concluding comments, workshop close

Module 1 / slide 10

Visual aids
Module 2

Concepts and principles of GM food safety assessment

GM food safety assessment

Presentation module 2

Concepts and principles of GM food safety assessment

Food and Agriculture Organization of the United Nations

GM food safety assessment

Presentation objectives

- Introduce the definitions, concepts and principles currently applied for the safety assessment of GM foods
- Introduce internationally agreed texts, guidelines and recommendations required for the safety assessment procedure

Module 2 / slide 2

GM food safety assessment

Definition: modern biotechnology

- The application of:
  - *in vitro* nucleic acid techniques, including r-DNA and direct injection of nucleic acid into cells or organelles,

  or
  - fusion of cells beyond the taxonomic family, to overcome natural physiological reproductive or recombinant barriers and using techniques not used in traditional breeding and selection

(Cartegena Protocol on Biosafety)

Module 2 / slide 3

GM food safety assessment

Modern biotechnology

- Broadens the scope of genetic changes
- Should not result in foods that are less safe than those produced by conventional techniques (OECD, 1993)

↓

- A new or different standard of safety is not required
- Previously established principles for assessing food safety still apply

Module 2 / slide 4

GM food safety assessment

International efforts

- Concerted efforts made internationally
- Key international consultations addressing the safety assessment of GM foods:
  - FAO/WHO
  - IFBC
  - ILSI
  - OECD
  - CAC
  - etc.
- The criteria used to assess the safety of GM foods are generally consistent from one country to another (World Bank, 2003)
- Countries may differ in statutory and non-statutory approaches to regulating GM foods

Module 2 / slide 5

GM food safety assessment

Key considerations

- International discussions between OECD countries, and within the United Nations FAO/WHO expert consultations, have resulted in a consensus on the specific safety issues that should be considered when evaluating a novel food

Module 2 / slide 6

Visual aids
Module 2 (cont.)

GM food safety assessment

General principles

- The following are used internationally in safety assessment of r-DNA foods:
  - conventional foods are generally considered to be safe, if provided prepared and handled
  - novel foods, including r-DNA foods, are required to undergo mandatory pre-market safety assessment in some jurisdictions (e.g. Japan, Canada, Australia and New Zealand, UK, EU)
  - an explicitly cautious approach is applied to foods, such as r-DNA foods, with no history of safe use

(cont.)

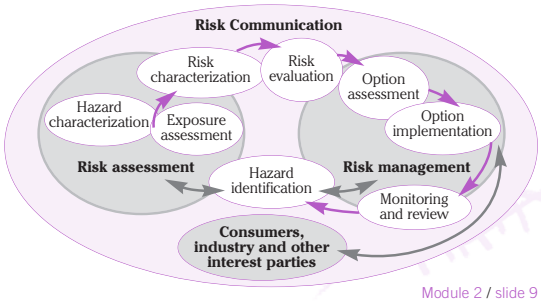
GM food safety assessment

General principles *(cont.)*

- Safety assessments of r-DNA foods are undertaken according to key principles:
  1. Safety assessments use scientific, risk-based methods.
  2. Safety assessments are conducted on a case-by-case basis.
  3. Both intended and unintended effects of genetic modification are considered.
  4. Where appropriate, comparisons are made with conventionally produced foods.
- Decisions with respect to safety are based on the totality of the evidence

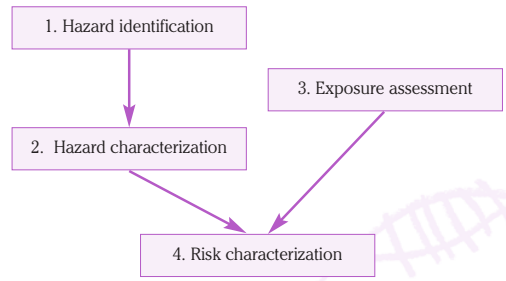
GM food safety assessment

Risk analysis framework



GM food safety assessment

Risk assessment



GM food safety assessment

1. Safety assessments use scientific, risk-based methods

- **Risk assessment** is the process of determining as accurately as possible the actual likelihood and consequences of the risks presented by exposure to identified hazards
- The objective is to **identify the potential for adverse effects** that r-DNA foods may pose for human health
- Use a modified **hazard identification scheme** referred to as a safety assessment to identify whether a hazard is present in the whole food

GM food safety assessment

2. Safety assessments conducted on a case-by-case basis

- Applied to a food commodity, for the food and food products derived from that modified commodity e.g. corn (kernels, corn flour, corn syrup, oil); canola (oil); cotton (oil and linters)
- Foods derived from a commodity (e.g. soybeans) that have been modified with different traits are assessed separately
- Any subsequent use of modern biotechnology requires a separate safety assessment

Visual aids
Module 2 (cont.)

GM food safety assessment

3. Consideration is given to both intended and unintended effects

Safety considerations apply to all aspects of the r-DNA food. Conducted in two phases:

1. Identification of similarities and differences
- traditional vs novel sources of donor DNA/genes
- molecular characterization – new genes, proteins, genetic stability
- compositional analysis

(cont.)

Module 2 / slide 13

GM food safety assessment

3. Consideration is given to both intended and unintended effects *(cont.)*

2. Identified differences are subjected to further scrutiny
- toxicity/allergenicity of any new protein
- safety of any transferred antibiotic resistance genes
- safety, nutritional impact and pattern of any compositional changes

Module 2 / slide 14

GM food safety assessment

4. Comparisons are made with conventionally produced foods

- r-DNA food variety compared with conventional counterpart food with history of safe use
- Comparison used to identify differences in levels of naturally occurring allergens, toxins, nutrients and antinutrients, or the ability to promote typical growth or well-being
- Significant differences (r-DNA vs conventional) assessed for biological significance and potential adverse health effects

Module 2 / slide 15

GM food safety assessment

Safety considerations

1. Description of the host organism that has been modified, including information on nutrient composition, known antinutrients, toxicants and allergenic potential, and any significant changes in these that may result from normal processing.
2. A description of the donor organism, including any known associated toxicity and allergenicity, and the introduced gene(s).
3. Molecular characterization of the genetic modification, including a description of the modification process and the stability of the introduced trait.

(cont.)

Module 2 / slide 16

GM food safety assessment

Safety considerations *(cont.)*

4. Identification of the gene products, including a description of the characteristics of the inserted gene.
5. Evaluation of the safety of expected new substances in the food, including an evaluation of any toxins produced directly by the modification.
6. Assessment of the new food's potential allergenicity.

(cont.)

Module 2 / slide 17

GM food safety assessment

Safety considerations *(cont.)*

7. Evaluation of the unintended effects on food composition, including:
   a) assessment of the changes in the concentration of nutrients or naturally occurring toxicants
   b) identification of antinutrient compounds that are significantly altered in novel foods
   c) evaluation of the safety of compounds that show a significantly altered concentration.

Module 2 / slide 18

Visual aids
Module 2 (cont.)

GM food safety assessment

Key initiatives:
to identify and address future needs

OECD task force for safety of novel foods and feeds

- consensus documents that provide guidance on critical parameters (e.g. key nutrients) of food safety and nutrition for each food crop
- documents for those products that have already been approved, as well as for commodities that are likely to be approved in the future
- http://www.oecd.org/document/63/0,2340,en_2649_34391_1905919_1_1_1_1,00.html

Module 2 / slide 19

GM food safety assessment

Key initiatives:
to identify and address future needs

Codex *ad hoc* intergovernmental task force on foods derived from biotechnology

- general principles for the risk analysis of foods derived from recombinant DNA plants
- guideline for the conduct of safety assessment of foods derived from recombinant DNA plants and microorganisms
- and more…
- http://www.fao.org/ag/agn/agns/biotechnology_codex_en.asp

Module 2 / slide 20

GM food safety assessment

Codex guideline

Codex guideline for foods derived from recombinant DNA plants

- The safety assessment of a food derived from a recombinant-DNA plant follows a stepwise process of addressing relevant factors including:
  - description of the r-DNA plant
  - description of host plant and its use as food
  - description of donor organism(s)
  - description of the genetic modification(s)
  - characterization of the genetic modification(s) *(cont.)*

Module 2 / slide 21

GM food safety assessment

Codex guideline *(cont.)*

- Safety assessment
  - expressed substances (non-nucleic acid substances): assessment of potential toxicity and assessment of possible allergenicity (proteins)
  - compositional analyses of key components
  - evaluation of metabolites
  - food processing
  - nutritional modification
  - other considerations (e.g. marker genes)

Module 2 / slide 22

GM food safety assessment

Key initiatives:
to identify and address future needs

FAO/WHO expert consultations

- Safety aspects of genetically modified foods of plant origin (June 2000)
- Allergenicity of genetically modified foods (January 2001)
- Safety assessment of foods derived from genetically modified microorganisms (September 2001)
- Safety of food derived from transgenic fish (November 2003)

(cont.)

Module 2 / slide 23

GM food safety assessment

Key initiatives:
to identify and address future needs *(cont.)*

- Safety of food derived from biotechnology
- FAO capacity building project to assist countries in implementing international standards related to the risk analysis of products derived from biotechnology
- http://www.fao.org/ag/agn/agns/biotechnology_en.asp

Module 2 / slide 24

Visual aids
Module 2 (cont.)

GM food safety assessment

Conclusions

- Concepts and principles developed by OECD, FAO/WHO and Codex have been practically applied by countries assessing the safety of foods derived from modern biotechnology
- Internationally conducted evaluations of r-DNA plant products have demonstrated that the concepts can be applied effectively in the safety assessment and approval of foods derived from modern biotechnology

(cont.)

Module 2 / slide 25

GM food safety assessment

Conclusions *(cont.)*

- The approach to the safety assessment of foods derived from modern biotechnology is continually evaluated, elaborated and expanded upon in international fora
- Participating countries contribute to the process and adopt updated approaches into their respective regulatory systems

Module 2 / slide 26

Visual aids
Module 3

The approach and framework for safety assessment of GM foods

GM food safety assessment

Presentation module 3

The approach and framework for the GM food safety assessment

Food and Agriculture Organization of the United Nations

GM food safety assessment

Presentation objective

- Introduce the concept of "comparative approach" and the reasons why the approach is used for safety assessment of GM foods
- Introduce the concept of "substantial equivalence (SE)", examples of tests using SE, issues regarding SE application, and background to adaptation of the concept
- Introduce Codex framework
- Explain stepwise approach and specific data requirements

Module 3 / slide 2

GM food safety assessment

Comparative approach

- Based on **the principle**: the products can be compared with conventional foods
- **Objective**: to determine if the GM food presents any new/altered hazard in comparison with its conventional counterpart
- **Goal**: not to establish an absolute level of safety, but rather the relative safety of the new products and that there is a reasonable certainty that no harm will result from the intended uses

Module 3 / slide 3

GM food safety assessment

Focuses

- Safety assessment is based on a comparison of an r-DNA organism to a counterpart or control with a history of safe use
- The focus of the comparison is to determine similarities and differences

Module 3 / slide 4

GM food safety assessment

Key concept

- If a new or altered hazard, or nutritional or other safety concern (noting that not every hazard is new, as many are present in the existing food) is identified, the risk should be characterized for its relevance to human and livestock health

Module 3 / slide 5

GM food safety assessment

Familiarity and determination

- **Familiarity** is defined as knowledge of the characteristics of a species and experience with the use of that species
- The **determination** is based on scientific literature and practical experience with the organism and similar varieties/lines

Module 3 / slide 6

Visual aids
Module 3 (cont.)

GM food safety assessment

Identifying unintended effects

- New products with intentionally altered nutritional profiles challenge the ability to assess unintended consequences
- Examples:
  - GM low-glutelin rice (decrease in glutelin levels was associated with an unintended increase in levels of prolamins)
  - GM Golden Rice (intentionally increased levels of beta-carotene, but unexpectedly found the modification accompanied by higher levels of xanthophylls)

Module 3 / slide 7

GM food safety assessment

Substantial equivalence

- First described in an OECD publication in 1993
- 60 experts, 19 countries, more than 2 years discussing how to assess the safety of GM foods
- Substantial equivalence was further endorsed by FAO/WHO joint expert consultation in 1996
- The concept of familiarity was the progenitor of the concept of "substantial equivalence", an approach developed by some jurisdictions as part of the risk assessment process

Module 3 / slide 8

GM food safety assessment

Key concept

- Substantial equivalence is one of many tools in the regulatory process for making decisions about particular characteristics of an r-DNA organism compared with its unmodified counterpart (e.g. a parent or host or donor)
- The concept should be used as a starting point to determine the safety of the differences found in the thorough analysis of an r-DNA organism, and not as a final decision step

Module 3 / slide 9

GM food safety assessment

Subject of debate

The appropriate use of the concept of substantial equivalence has been the subject of much debate by many expert bodies

- OECD, FAO/WHO, Codex
- Institute of Food Technology, 2000
- NZ Royal Commission on Genetic Modification, 2001
- Canadian Royal Society Report, 2001
- US NAS, 2002
- UK Royal Society, 2002
- Society of Toxicology Position Paper, 2002
- American College of Nutrition, 2002
- UK Government Report, 2003

Module 3 / slide 10

GM food safety assessment

Adoption of the concept

- The **OECD** and some other international organizations recognize it as a valid concept that "contributes to a robust safety assessment framework"
- The **Codex Task Force** continues to work with the concept of substantial equivalence in safety and nutritional assessment and to speculate about alternative strategies
- The **Codex Guideline** includes reference to substantial equivalence (paragraph 13) as a key step in the safety assessment process, not a safety assessment in itself

Module 3 / slide 11

GM food safety assessment

Limitations of substantial equivalence

- Requires sufficient analytical data to be available in the literature, or be generated through analysis
- Dependence on a comparator and on the information that is available, or can be generated for the comparator
- The choice of comparator is crucial to effective application of the concept
- An appropriate comparator must have a well-documented history of use

Module 3 / slide 12

Visual aids
Module 3 (cont.)

GM food safety assessment

Remarks on the comparative approach

- **Whole food** vs **individual substances** (additives, pesticides, etc.)
  - safety assessments require different approaches
  - whole foods from many crops sometimes contain natural toxicants, antinutrients – may be important to the plant but may be harmful to humans
- **Codex Guideline** recommends that a **comparative assessment** be used to determine the safety of GM food (as safe as conventional counterpart)
  - paragraphs 13–17

Module 3 / slide 13

GM food safety assessment

Codex framework

- "Principles for the risk analysis of foods derived from modern biotechnology" (2003)
- "Guideline for conduct of food safety assessment of foods derived from recombinant-DNA plants" (2003)

Module 3 / slide 14

GM food safety assessment

Stepwise approach

- Guideline **paragraphs 18–21**
- **Goal**: to examine the intentional and unintentional consequences of the specific modification on food components, in comparison with a counterpart food that has a history of safe use

Module 3 / slide 15

GM food safety assessment

Specific data requirements

- Description of the recombinant-DNA plant (paragraph 22)
- Description of the host plant and its use as food (paragraphs 23–25)
- Description of the donor organism(s) (paragraph 26)
- Description of the Genetic Modification(s) (paragraphs 27–29)

Module 3 / slide 16

GM food safety assessment

Working group assignment

- Using case studies, **identify** the following:
  - description of the recombinant-DNA plant
  - description of the host plant and its use as food
  - description of the donor organism(s)
  - description of the genetic modification(s)
- Then **review** the sufficiency of information for the above items

Module 3 / slide 17

Visual aids
Module 4

Characterization of GM, assessment of possible toxicity, possible allergenicity and compositional analysis

GM food safety assessment

Presentation module 4

Characterization of GM, assessment of possible toxicity & allergenicity and compositional analysis

Food and Agriculture Organization of the United Nations

GM food safety assessment

Presentation objectives

- Explain the methodology of characterization of the genetic modification(s)
- Introduce methodology of toxicity assessment including animal studies
- Introduce methodology of potential allergenicity assessment including important parameters
- Introduce methodologies of compositional analysis, evaluation of metabolites, food processing and nutritional modification

Module 4 / slide 2

GM food safety assessment

Characterization of the Genetic Modification(s)

- Codex Guideline paragraphs 30-33
  - Molecular analysis
  - Randomly generated plant transformation events
  - Transgene detection using event-specific primers
  - Extent of refinement at the current level of the technology

Module 4 / slide 3

GM food safety assessment

Toxicity

- Codex Guideline paragraphs 34-40
- Key considerations:
  - protein expression product(s) of the inserted gene(s)
  - effects resulting from disruption of gene expression due to insertion of donor DNA into the host genome
- Intention to determine safety: as safe as the conventional counterpart
  - e.g. conventional soybean has the potential to affect endocrine functions – GM soybean with an equivalent composition would have the same potential

Module 4 / slide 4

GM food safety assessment

In vitro studies

- Novel proteins (as opposed to other chemicals)
  - predictable metabolic fate in the human/animal gut
  - *in vitro* digestibility assay – indicates the likelihood of a protein having characteristics unusual for dietary proteins
- If a protein is shown to be resistant to typical digestive fluid: significant for proteins with potentially adverse biological activities (toxicity or allergenicity)
- Proteins that exhibit toxicity generally exert their effect in a short time frame – acute toxicity tests are considered adequate

Module 4 / slide 5

GM food safety assessment

Animal studies

- Major element of the safety assessment of many compounds: pesticides, pharmaceuticals, chemicals, food additives, etc.
- Substance: known purity, no nutritional value
- Codex Guideline paragraphs 10–12 and 53
- Difficulties: foods are complex mixtures of compounds in composition and nutritional value – identifying potential adverse effects in animal studies without appropriate control treatments is extremely difficult

Module 4 / slide 6

Visual aids
Module 4 (cont.)

GM food safety assessment

Allergenicity (proteins)

- Codex Guideline paragraphs 41–43
- True food allergies may involve several types of immunological responses
- Most common types: allergen-specific immunoglobulin E (IgE) antibodies

(cont.)

Module 4 / slide 7

GM food safety assessment

Allergenicity (proteins) *(cont.)*

- True food allergies may also include cell-mediated reactions
- Codex has adopted a list of the most common allergenic foods associated with IgE-mediated reactions
- GM food crops can introduce potential allergenicity into the human diet
- Codex recommends that an integrated, stepwise, case-by-case approach be used in the assessment of possible allergenicity of GM food

Module 4 / slide 8

GM food safety assessment

Assessment strategy

- 1st step – the determination of:
  - the source of the introduced protein
  - any significant similarity between the amino acid sequence of the protein and that of known allergens
  - its structural properties
- No single test can predict the likely human IgE response to oral exposure
- Isolation of any newly expressed proteins from the r-DNA plant in order to characterize the protein
- Important to establish whether the source is known to cause allergic reactions

Module 4 / slide 9

GM food safety assessment

Important parameters

- Source of the protein
- Amino acid sequence homology
- Pepsin resistance
- Specific serum screening

Module 4 / slide 10

GM food safety assessment

Remarks on toxicity and allergenicity assessment

- **Quality assurance**
  - It is very important that the organizational process and the conditions under which lab studies are planned, performed, monitored, recorded & reported are according to the principles of GLP
  - Toxicology studies: it is important to establish the relationship of changes in physiological parameters measured to the dose levels of the tested compound

(cont.)

Module 4 / slide 11

GM food safety assessment

Remarks on toxicity and allergenicity assessment *(cont.)*

- **Other methods & tools**
  - As scientific knowledge and technology evolve, other methods and tools may be considered in assessing the allergeniciy potential of newly expressed proteins

Module 4 / slide 12

Visual aids
Module 4 (cont.)

GM food safety assessment

Compositional analysis

- Both beneficial and harmful components in the human diet:
  - nutrients
  - bioactive non-nutrients
  - antinutrients
  - toxicants
  - contaminants
  - other potentially useful and dangerous elements
- It is important to decide which nutrients/elements to focus the evaluation on
- Codex Guideline paragraphs 44–46

Module 4 / slide 13

GM food safety assessment

Food processing

- Codex Guideline paragraph 47
- Processing methods can cause a significant variation in the nutrient content of a food
- Modern separation techniques (milling, centrifugation, pressing, etc.) change the nutritional content
- Heating techniques may reduce the content of many heat-labile nutrients (vitamins, phytochemicals, etc.)

Module 4 / slide 14

GM food safety assessment

Nutritional modification

- Codex Guideline paragraphs 48-53
- For r-DNA plants that were intentionally developed to have altered nutrients, the aim of the nutritional evaluation is to demonstrate that there are no unintentional changes (bioavailability etc.)
- The compositional differences are likely to be greater – thus current methods for safety assessment may be found limiting, due to that nutritionally modified crops will not be substantially equivalent to their conventional counterparts, and share fewer compositional values for comparison

Module 4 / slide 15

GM food safety assessment

New analytical methods

- Further improvement of methodologies and more sensitive techniques allow detection of unintended alterations in the composition that were once undetectable
- The utility and applicability of the non-targeted techniques for risk assessment need further exploration in validating the relevance to food safety of observed changes
- Profiling methods are not yet suitable for risk assessment purposes, but if validated they may be useful to confirm and supplement other data

Module 4 / slide 16

GM food safety assessment

Working group assignments

- Using case studies:
  - **identify** the toxicity studies, and **evaluate** the possible toxicity
  - **identify** the allergenicity studies, and then **evaluate** the possible allergenicity
  - **identify** the description of compositional analysis, and then **evaluate** the analysis

Module 4 / slide 17

Visual aids
Module 5

Risk communication and safety assessment decisions

GM food safety assessment

Presentation module 5

Risk communication and safety assessment decisions

Food and Agriculture Organization of the United Nations

GM food safety assessment

Presentation objectives

- Introduce risk communication in the context of risk analysis (Codex)
- Explain what risk communication should and should not be
- Explain the patterns of risk perception and trust
- Introduce a food safety-related communication strategy
- Introduce FAO/WHO expert consultation recommendations

Module 5 / slide 2

GM food safety assessment

Risk communication in the context of risk analysis

- Working Principles for Risk Analysis for Application in the Framework of the Codex Alimentarius (2003): general orientation on risk analysis
- Communication linked to the risk analysis process
- Embedded in risk assessment and risk management
- Active at the start of the risk analysis process – not an add-on at the end
- Everyone's responsibility

Module 5 / slide 3

GM food safety assessment

Working principles: general aspects

- **Risk analysis process**:
  - applied consistently
  - open, transparent and documented
  - **the three components of risk analysis to be documented fully and systematically in a transparent manner**
  - **effective communication and consultation with interested parties throughout risk analysis**
- **Structured approach**:
  - Risk assessment, risk management and risk communication, each of the three components being integral to the overall process and applied in an overarching framework

Module 5 / slide 4

GM food safety assessment

Codex definition of risk communication

- The interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions

Module 5 / slide 5

GM food safety assessment

Risk communication should:

- promote awareness and understanding of the specific issues under consideration
- promote consistency and transparency in formulating risk management options and recommendations
- provide a sound basis for understanding the risk management decisions proposed
- improve the overall effectiveness and efficiency of the risk analysis

(cont.)

Module 5 / slide 6

Visual aids
Module 5 (cont.)

GM food safety assessment

Risk communication should *(cont.)*:

- strengthen the working relationships among participants
- foster public understanding of the process, so as to enhance trust and confidence in the safety of the food supply
- promote the appropriate involvement of all interested parties
- exchange information in relation to the concerns of interested parties about the risks associated with food

Module 5 / slide 7

GM food safety assessment

Risk communication involves…

- a two-way process
- understanding people's perception of risk
- opportunities for public involvement in decision making
- timely and accurate information
- internal communication

Module 5 / slide 8

GM food safety assessment

Risk communication is not…

- just about communicating risk
- simply selling decisions to the public
- a crisis-related process
- the sole responsibility of communication specialists

Module 5 / slide 9

GM food safety assessment

Major function of risk communication

- To ensure that all information and opinion required for effective risk management is incorporated into the decision-making process
- Should include a transparent explanation of:
  - the risk assessment policy
  - the assessment of risk
  - including the uncertainty

Module 5 / slide 10

GM food safety assessment

As an important part of biosafety procedure

- To ensure public acceptance of food derived from recombinant-DNA plants
  - communicate and interact with the public about the specific risks and actions taken
  - a mechanism that builds confidence among the stakeholders in a gradual manner moving along with the different phases of the development of the r-DNA plant and foods derived from it

Module 5 / slide 11

GM food safety assessment

Two components of risk communication

- **technical components**, which generally concern the scientific hazards evaluated in the **risk assessment** and **management options** arising out of the assessment
- **non-technical components**, which include the **administrative** protocols, and the **cultural** and **ethical** issues in the society dealt with by the regulatory agencies during the process of risk analysis

Module 5 / slide 12

Visual aids
Module 5 (cont.)

GM food safety assessment

Regulatory risk communication

- Codex principles paragraphs 22–24
  - explain both how and why decisions are taken
  - acknowledge any concerns raised by stakeholders
  - explain how these concerns have been addressed
- A number of countries have adopted OECD measures for information dissemination
  - inviting public comments on safety evaluation reports
  - disclosure of data used in safety assessments
  - publication of results of meetings of safety assessment bodies

Module 5 / slide 13

GM food safety assessment

Risk communication as a two-way process

- Regulatory risk communication
  - providing information about the regulatory framework and processes
  - gathering input and feedback
- Credibility is built into the communication process by providing technical reviews on the process in simple language
- Two questions that need to be answered - raise the issue of choice and knowing what foods from r-DNA plants may be in the marketplace:
  - are foods from r-DNA plants safe?
  - what foods have been genetically modified?

Module 5 / slide 14

GM food safety assessment

Perception of risk

- We all see the world differently (mindsets)
- People of similar backgrounds tend to perceive risk in a similar way
- Some gender differences
- People with less control over their lives tend to see greater risk
- Evidence-based perception of risk: **RISK=HAZARD**
- Consumer perception of risk: **RISK=HAZARD+OUTRAGE**

Module 5 / slide 15

GM food safety assessment

Consumers' perceptions of levels of risk

- Media coverage can often be alarmist
- Stakeholders concerned about balancing health messages with potential risks
- Applies to many contaminants in food issues
- The acceptable level of risk differs between countries and communities

Module 5 / slide 16

GM food safety assessment

Trust

- Public confidence in the safety of the food supply
- Trust in industry and government regulators to ensure safe food
- Hard to regain trust once it is lost
- Not a level playing field
- Negative events are more noticeable than positive events
- Sources of bad news are seen as more credible
- The media is attracted to bad news
- Special interest groups are skilful in using the media

Module 5 / slide 17

GM food safety assessment

Information release

- Early release is the key
  - story will leak anyway – loss of trust/credibility
  - people entitled to information affecting lives
  - sets pace for resolution of issue
  - better control of accuracy of information
  - less work to release than respond to inquiries
  - less chance of public becoming angry
  - less chance public will over-estimate risk
  - more time for public involvement

(Adopted from New Jersey Department of Environmental Protection, 1987)

Module 5 / slide 18

Visual aids
Module 5 (cont.)

GM food safety assessment

Communication strategy

Risk	*Perceived risk*	*Examples*	*Strategy*
Low	Low	Allowed level of contaminants	Passive
Low	High	**GM foods**, dioxins, mercury in fish	Responsive
High	Low	Microbial contamination	Educative
High	High	BSE	Proactive

(cont.)

Module 5 / slide 19

GM food safety assessment

Communication strategy *(cont.)*

- Identify audiences – segment stakeholder groups (don't forget internal audiences)
- Prepare messages – normally three key messages and separate messages to each audience
- Select communication tools

Module 5 / slide 20

GM food safety assessment

Media

- Press, radio and television
- Establish working relationships and credibility in non-crisis times
- Know what messages you want to convey
- Be open and honest… and available
- Be helpful
- Understand how the media works

Module 5 / slide 21

GM food safety assessment

FAO/WHO: useful considerations in risk communication

- Know the audience
- Involve the scientific experts
- Establish expertise in communication
- Be a credible source of information
- Share responsibility
- Differentiate between science and value judgement
- Assure transparency

Module 5 / slide 22

GM food safety assessment

Risk communication in safety assessment

- Creation of better information about the regulatory system
- Creation of a centralized information body
- Increase public awareness and engagement

Module 5 / slide 23

GM food safety assessment

Working group assignments

Using case studies:

- **strategize** the methods of risk communication
  - involving all the stakeholders?
  - having a good relationship with the media?
  - building credibility?
- **prepare** a decision document for the general public

Module 5 / slide 24

Part Three

Case studies

In order to enhance the utility of the case studies for training purposes, certain information has been summarized and the data presented in the case studies are only a subset of those actually submitted. The case studies do not reflect a complete application, nor a complete safety assessment. These case studies are included in this training package without any modification or enhancement by FAO. The views expressed in the case studies do not necessarily reflect the views of FAO.

Case study 1

Food safety assessment of genetically modified insect resistant corn event MON 810

Preface

The United States Food and Drug Administration (FDA) completed a consultation for insect resistant (protected) corn line MON 810 in 1996. Health Canada notified Monsanto that the Department had no objection to the food use of corn line MON 810 in 1997. These decisions were made by both regulatory authorities following a comprehensive assessment of MON 810 based upon internationally accepted principles for establishing the safety of foods derived from genetically modified plants. The record of review and decision-making is described for the FDA consultation in Appendix 1 and for Health Canada's assessment in Appendix 2.

The data and information in this case study have been summarized for training purposes. The case study is derived from parts of the food safety submission assessed by Health Canada. Monsanto Canada Inc. provided data on the description of the new variety, the donor organism(s), the genetic modification methods and characterization. The novel protein was identified, characterized and compared to the original bacterial protein, including an evaluation of its potential toxicity. Scientific publications and data from field testing in Canada and the United States under confined trials in 1995 and 1996 were supplied.

Note that statements in quotes are taken directly from the submission to Health Canada.

Disclaimer

Monsanto Canada Inc. has consented to the use of the information provided in their regulatory submission for event MON 810 as a training tool. It must be noted, however, that in order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original applications. Certain information has been reduced to summaries and the present data as presented in the case study are only a subset of that actually submitted. The case study in no way constitutes a complete application nor is it to be considered a complete safety assessment. To that end, the use of this information in the form of a training tool does not constitute an endorsement of the information or product nor should it be considered a reflection of any of the original submissions.

Description of the recombinant-DNA plant

Line MON 810 contains an inserted genetic fragment of the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 that produces an active delta endotoxin protein expressed in the corn tissue. The target pest, European corn borer (ECB) (*Ostrinia nubilalis*), is an important corn insect pest. Physical damage is caused by ECB feeding on various tissues of the corn plant. The tissues damaged depend on the number of generations of ECB. The damage from ECB feeding includes: a) leaf feeding, b) stalk tunneling, c) leaf sheath and collar feeding, and d) ear damage. Estimated losses range from 5-10% corn yield annually from ECB from disruption of nutrient and water translocation, secondary disease infections, stalk lodging, ear droppage and kernel damage.

The company further describes the variety and its history, "Line MON 810 was supplied to various seed companies as F1 seed of transformed genotype Hi-II crossed to several various elite inbreds. The resulting lines were subjected to multiple cycles of backcrossing to the recurrent inbred parent to recover the converted elite genotype, followed by several cycles of selfing to derive converted inbred parents for hybrid testing. Further cycles of seed increase (selfing) are required to produce parent seed for commercial hybrid seed production. Insect-protected hybrid seed will be heterozygous for the *cryIA(b)* gene since one inbred parent containing the gene is sufficient to confer the insect-protected phenotype on progeny hybrids."

MON 810 is a field corn, not a sweet corn and is intended primarily as an animal feed, but some human food uses occur for field corn. For example, MON 810 may be used either dry or wet milled in processed corn products for humans. No differences in the intended uses of MON 810 are expected as compared to existing field corn hybrids.

Description of the host plant and its use as food

The host plant used is a hybrid line of *Zea mays* with a Mo17X (Hill X B73) background. These corn lines have a long history of use in particular as animal feed, being field corn and not sweet corn.

Zea mays L. (corn, maize) has been cultivated for over 8000 years in Mexico and Central America. A versatile and responsive species, corn has increased both in productivity and geographical range over the past century

with the development of hybrids, breeding programs and fertilizer use and is now grown on every habitable continent. Corn yields prior to hybridization in the early 1930s were around 1.3 metric ton per hectare (ha). The current record high is 123.5 t/ha (with an average of around 137 bushels per acre in the US). World production of corn in 2000 is estimated at 23,800 million bushels.

Corn is used for many different products and uses, as a staple food in many parts of the world and in derived forms, such as starch, alcohol, oil, and for animal feed. Also, corn is used for production of ethanol as a renewable fuel.

Description of the donor organism(s)

The donor of the *cryIA(b)* gene that codes for the CryIA(b) protein, a delta endotoxin active against lepidopteran insect pests, is *Bacillus thuringiensis* subsp. *kurstaki* (B.t.k.) strain HD-1.

The cryIA(b) gene inserted into MON 810 originates from a *Bacillus thuringiensis* subsp. *kurstaki*. *Bacillus thuringiensis* (or Bt) species are spore-forming, gram-positive bacteria that produce a crystal with insecticidal properties. Bt species have been used commercially as pest control agents for decades.

Different strains of Bt are insecticidally active against selected insect pests:

- Bt *israelensis* strains for dipterans (mosquitoes and black flies)
- Bt var. *sandiego* and *tenebrionis* strains for coleopterans (Colorado potato beetle, elm leaf beetle, yellow mealworm)
- Bt *kurstaki*, *thuringiensis*, *sotto* and *aizawai* strains for lepidopterans (corn borer, tomato hornworms, gypsy moth, cabbage looper, tobacco budworm, cotton bollworm).

The delta endotoxin crystals are produced when the bacterium sporulates. To be active, the protein must be ingested by the insect. While the protein is insoluble at neutral or acidic pH, it is soluble at the alkaline pH that occurs in the guts of larval insects where it is activated by proteases in the gut. The activated protein (stripped of its carboxy terminal and about 28 amino acids from the amino terminal end, at approximately 600 amino acids in size) diffuses through the peritrophic membrane of the insect to the midgut epithelium. There it binds to the specific high affinity receptors on the surface of the insect midgut, inserts itself into the membrane and forms ion-specific pores (non-target insects, birds, mammals and fish do not have these receptors). The resulting pores in the membrane cause leakage of the intracellular contents into the gut lumen and water into the epithelial gut cells which swell and lyse. The gut becomes paralyzed disrupting the digestive process, which causes the insect to stop eating and die.

The protein produced in MON 810 insect protected (IP) corn is identical to that produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, which controls insect pests by the production of delta-endotoxin crystals. Data to support this claim are supplied in the submission.

B.t.k. has been used as a microbial pest control agent for decades and "the naturally occurring Bt proteins have been demonstrated to be virtually non-toxic to fish, avian species, mammals and other nontargets … no adverse effects are expected to wildlife from the commercialization of these plants."

The company's submission states: "The CryIA(b) protein is insecticidal only to lepidopteran insects. Only seven of the eighteen insects screened were sensitive … and they were all lepidopteran. This specificity is directly attributable to the presence of receptors in the target insects. Selective activity of B.t.k. endotoxin will not disrupt populations of either beneficial insects or nontarget animals (*e.g.*, birds, fish)."

Tests (cited from the literature), registration documentation and safety assessments from pesticidal registrations on commercially available microbial pesticide products, such as DIPEL®, indicate that they are "widely recognized as nontoxic for mammals, birds and fish as well as beneficial nontarget insects including predators and parasitoids of lepidopteran insect pests and honeybee."

Description of the genetic modification

Plasmid DNA was introduced into the plant tissue by particle acceleration (also known as biolistic transformation). The DNA is precipitated onto the surface of microscopic tungsten or gold particles using calcium chloride and spermidine. A drop of coated particles, placed onto a plastic macrocarrier, is accelerated at high velocity through a barrel by a gunpowder explosion. The macrocarrier flight is stopped by a plastic stopping plate allowing the DNA-coated particles to continue their journey, penetrating plant cells in the path of the explosion. The DNA is deposited and incorporates into the cell chromosome. The cells are incubated on a tissue culture medium containing 2,4-D, which supports callus growth. The cells with introduced DNA contain genes for glyphosate tolerance and are

grown in the presence of glyphosate to select the transformed cells.

Two plasmids were used during this biolistic process, PV-ZMBK07 (Figure 1) containing the cryIA(b) gene and PV-ZMGT10 (Figure 2) containing two marker genes used for selection on glyphosate, CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) and glyphosate oxidoreductase (gox). Tables 1 and 2 describe the DNA elements in the plasmids.

Only a portion of the PV-ZMBK07 plasmid vector is present in MON 810 and the final MON 810 construct does not contain the marker genes. Details on how this was determined follow in Chapter 3. "It is presumed that the genes which allow for selection on glyphosate were originally incorporated into the plant genomic DNA but were lost by segregation during backcrossing." The reason given is that these genes "integrated at a separate loci from the cryIA(b) gene and segregated out during the crossing."

While both plasmids contain the nptII gene encoding for neomycin phosphotransferase II (*nptII*) under the control of its own bacterial promoter, data shows that the *nptII* gene is not present in MON 810. This bacterial gene was used as a selectable marker during plasmid construction.

Experiments in corn transformation have demonstrated that the frequency of obtaining transformants containing glyphosate tolerance selection

Table 1. Summary of DNA elements in plasmid PV-ZMBK07 (See Fig. 1)

Genetic element	*Size Kb*	*Function*
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
hsp 70 intron	0.80	Intron from the maize *hsp70* gene (heat shock protein) present to increase the level of gene transcription
cryIA(b)	3.46	The gene encodes the CryIA(b) protein product
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
lacZ	0.24	A partial *E. coli lacI* coding sequence, the promoter P*lac* and a partial coding sequence for β-D-galactosidase or lacZ protein from pUC119
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in *E. coli*
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid

Table 2. Summary of DNA elements in plasmid PV-ZMGT10 (See Fig. 2)

Genetic element	*Size Kb*	*Function*
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
hsp 70 intron	0.80	Intron from the maize *hsp70* gene (heat shock protein) present to increase the level of gene transcription
CTP2	0.31	Chloroplast transit peptide (CTP) isolated from *Arabidopsis thaliana* EPSPS present to direct the CP4 EPSPS protein to the chloroplast, the site of the aromatic amino acid synthesis
CP4 EPSPS	1.4	The gene for CP4 EPSPS, isolated from *Agrobacterium* sp strain CP4 which allows for the selection of transformed cells on glyphosate
CTP1	0.26	Chloroplast transit peptide (CTP) isolated from the small subunit gene of ribulose-1,5-biphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana* present to direct the GOX protein to the chloroplast, the site of the aromatic amino acid synthesis
gox	1.3	The gene encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase (GOX) isolated from *Achromobacter* sp. (new genus *Ochrobactrum anthropi*) strain LBAA
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
lacZ	0.24	A partial *E. coli lacI* coding sequence, the promoter P*lac* and a partial coding sequence for β-D-galactosidase or lacZ protein from pUC119
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in *E. coli*
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid

was increased when both plant selectable markers were used.

The plasmid size of PV-ZMBK07 is 7794 bp and of PV-ZMGT10 is 9427 bp.

Characterization of the genetic modification

Introduction

Several methods, including Southern and Western blot analyses, were used in the molecular characterization of MON 810. Possible novel genes and potential gene products that may have been present in MON 810, based on the information in the plasmid maps, are listed in Table 3.

Molecular characterization

Molecular characterization of the integrated DNA (I-DNA) included determination of:

- The insert number (number of integration sites within the corn genome)
- Copy number (number of each gene within the integrated DNA)
- Insert integrity.

Southern blot analysis was used to determine the above parameters.

MON 810 is compared against a non-transgenic control (counterpart) MON 818, which also has a Mo17 X (Hi-II X B73) background. MON 818 does not contain the genes encoding for B.t.k. HD-1 Cry1A(b), CP4 EPSPS or GOX proteins.

Table 3. Possible novel genes and potential gene products in MON 810.

Novel gene	*Novel gene product*	*Regulatory sequence*	*Other DNA sequences*
PV-ZMBK07			
crylA(b)	*Bt* gene	Sequence is controlled by E35S promoter (0.6Kb) and a 0.8 Kb intron from the hsp70 gene (heat shock protein) is present to increase the levels of gene transcription. A 0.24 Kb nopaline synthase 3' nontranslated terminator sequence (NOS 3') attached to the *cry* gene provides the mRNA polyadenylation signals.	
lacZ-alpha	Betagalactosidase. A polylinker (region with multiple cloning sites) which allowed the cloning of the desired genes in the plasmid vector	Bacteria controlled promoter. Joined at the 3'end of NOS.	Followed by a 0.7 Kb region of replication for the pUC plasmids (*oripUC*) which allows replication of plasmids in *E. coli*.
nptII (marker for selection during construction of the plasmid derived from procaryotic transposon Tn5)	Neomycin phosphotransferase Resistance to aminoglycoside antibiotics (*i.e.*, kanamycin and neomycin)	Has its own bacterial promoter	
PV-ZMGT10			
gox gene cloned from *Achromobacter* sp. strain LBAA	Glyphosate metabolizing enzyme, glyphosate oxidoreductase (GOX). Degrades glyphosate by conversion to aminomethylphosphonic acid and glyoxylate	Joined to CTP1 peptide which targets the gene to the plastids, a chloroplast transit peptide. Derived from a subunit of ribulose - 1,5 bisphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana*. Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	
CP4 EPSPS Isolated from *Agrobacterium* species strain CP4 which is resistant to glyphosate	5-enolpyruvylshimkimate-3-phosphate synthase	Joined to CTP2 peptide. Isolated from *Arabidopsis thaliana* EPSPS. The gene and CTP2 are about 1.7Kb in size. Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	
Also contains the same *lacZ-alpha*, ori-pUC and *nptII* genes described above			

Insert Number

After digestion of extracted DNA with restriction enzyme NdeI, which does not cleave within either of the plasmids used to produce MON 810, analysis shows that a single band at approximately 5.5 Kilobase (Kb) was observed (Figure 3). This indicates that the DNA from the plasmid was present at one site. The rationale for this is that since there are no restriction sites inside the plasmids, the enzyme cleaves outside the inserted DNA releasing a fragment containing the inserted DNA and some adjacent genomic DNA. Since the plasmid DNA inserts randomly in the DNA of the plant, the distance between the inserted DNA and the restriction enzyme sites in the plant DNA will vary. If there are multiple insertion sites it is likely that cutting with a restriction enzyme that cleaves only outside the insert, the released fragment containing the inserted DNA would vary in size depending on the distance from the NdeI retriction site. You would expect to see multiple bands detected in the Southern if there were multiple insertion sites.

Insert Composition

Using a number of probes, tests show that the CP4 EPSPS, gox and ori-pUC sequences were not detected in MON 810, whereas nptII, E35S, hsp70 and the cryIA(b) were present within the 5.5 Kb NdeI fragment.

cryIA(b)

Digestion of DNA with NcoI/EcoRI to release the cryIA(b) gene followed by Southern blot analysis found an approximately 3.1 Kb fragment (Figure 4), which is "sufficient to encode an insecticidally active CryIA(b) protein." While "the positive hybridization control (lane 1 of figure 4) produced one 3.46 Kb fragment which corresponds to the expected size of cryIA(b) gene, the MON 818 DNA (lane 2) does not contain any bands, as expected for the control line. The MON 810 DNA contains one band of approximately 3.1 Kb."

Western blots indicate that the trypsin resistant protein of 63 Kilo-Dalton (kD) is produced by the integrated partial cryIA(b) gene in MON 810 (Figures 5 and 6). "Based on the Western blot data and efficacy of maize line MON 810, the cryIA(b) gene present produces an insecticidal CryIA(b) protein which provides effective, season long control of ECB."

CP4 EPSPS

Digestion with NcoI/BamHI would release any CP4 EPSPS genes present. Southern blots (Figure 7) indicate that MON 810 does not contain the 3.1 Kb fragment (the expected size of CP4 EPSPS) found in the gel spiked with the two plasmids. The CP4 EPSPS protein was not detected by ELISA in leaf, whole plant or grain tissues. Western blot analysis confirms the absence of the protein from leaf extracts (Figure 8, lane 9).

gox

Digestion with NcoI/BamHI would excise the gox gene, if present (NcoI to NcoI) and would be about 3.1 Kb in size. Southern blot analysis (Figure 7) indicates that MON 810 does not contain the gox gene. Neither was it detected by ELISA of plant tissues nor by Western blot analysis (Figure 9, lane 8).

Plasmid backbone

In order to detect backbone (nptII/ori-pUC) DNA, the nptII gene was used to probe a NcoI/EcoRI digestion of the Mon 810 DNA and PV-ZMBK07 plasmid DNA. When probed with the nptII gene, Southern analysis detected bands only for the plasmid at 2.5 Kb and 1.8 Kb. No signal was detected in the MON 810 DNA. Using the ori-pUC DNA a 1.8 Kb band for detected in the plasmid lane, but the ori-pUC) Southern blots (Figure 10) indicate that MON 810 contains no ori-pUC backbone sequences.

From the above information the interpretation is that one I-DNA containing approximately 4 Kb of DNA from the PV-ZMBK07 plasmid consisting of a portion of the enhanced E35S promoter (estimated to include one of two enhancer elements plus the promoter), the full length intron from the hsp70 gene (heat shock protein) and 2448 bp of the full length of 3468 bp cryIA(b) gene was inserted in the genome of MON 810, as shown in the schematic in Figure 11. No DNA from the bacterial vector backbone (*e.g.*, the pUC-origin of replication), the nptII, gox or CP4 EPSPS genes was detected. The submission states that, "MON 810 contains one integrated DNA contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, maize hsp70 intron and the cryIA(b) gene." Western analysis established that the trypsin resistant 63 kD B.t.k. HD-1 protein was produced in MON 810.

CryIA(b) gene integrity and activity

During particle acceleration plasmid DNA can be broken, resulting in integration of partial genes into the genomic DNA. Southern blots and genomic clone sequence established that the first 2448 bp of the 3468 bp cryIA(b) gene integrated into MON 810.

Modified plant expression

Molecular analysis of MON 810 "established that the line only contains cryIA(b) gene from plasmid PV-ZMBK07 and not the CP4 EPSPS, gox or nptII/ori-pUC genes. There is no evidence that any of the DNA contained in plasmid PV-ZMGT10 was inserted. MON 810 contains one integrated DNA fragment, contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, the maize hsp70 intron and the cryIA(b) gene."

The 'cry1a(b)' gene and its novel trait

The full length gene encoding for CryIA(b) protein has been described. While the genes inserted into MON 810 have been modified to enhance expression in corn, the amino acid sequence of expressed protein is identical to natural protein derived from B.t.k. The *cryIA(b)* gene fragment (Table 4) inserted into the MON 810 has been shown to be equivalent to the original bacterium source, as far as activity against insect pests. Table 4 is a summary of the gene product and its characteristics as submitted by the company.

Western analysis was used:

- To assess the protein products of the partial gene using antibodies specific to B.t.k. proteins
- To compare them to the *E. coli* produced protein standard and tissue extracts from other insect protected corn lines
- To look for any anomalous or unexpected protein products (ex. CP4 EPSPS and GOX (Figures 8, 9, and 12)), and
- To determine if the expressed B.t.k. protein was converted to the expected size of 63 kD trypsin-resistant protein product (Figures 5 and 6).

The company stated, "as is commonly observed in Western blot analysis of Bt proteins, multiple protein products were observed for line MON 810 and the other six insect protected corn lines (Figure 5, lanes 5-11). The full-length gene was not observed in line MON 810, as expected since the full-length gene was not incorporated into the corn genome. … MON 810 showed no apparent differences in the size ranges of the less than full length protein products … when compared to the other six insect protected lines produced with the same full length *cryIA(b)* gene. The predicted molecular weight of the B.t.k. HD-1 protein from the partial *cryIA(b)* gene is 92 kD but is not detected, probably due to low expression or rapid degradation to the trypsin-resistant product during the extraction process."

When the protein extracts are subjected to trypsin digestion, all seven lines show the core protein at approximately 63 kD (Figure 6).

The protein products in MON 810 and expected immuno-reactive products are similar to those in other IP corn lines, except for the lack of the full length B.t.k. HD-1 protein. No unexpected products were observed. The trypsin results demonstrate that the partial *cryIA(b)* gene inserted into MON810 produces the efficacious trypsin-resistant B.t.k. HD-1 protein.

Equivalence of bacterial and plant produced protein

Escherichia coli containing the B.t.k. gene was used to produce the quantities of the CryIA(b) protein needed to do tests, such as feeding trials. Therefore, the equivalence of the B.t.k. HD-1 protein produced in the IP corn was assessed against that from the *E. coli*. As the company states, the rationale is that: "the expression level of B.t.k. HD-1 in IP corn plants is extremely low. Therefore it is not feasible to isolate this protein from plants in sufficient quantity to conduct the various safety studies performed for the registration of this product. The best alternative was to isolate the functionally active B.t.k. HD-1 protein produced in a microbial host … and verify its physical and functional equivalence to the plant-expressed protein. Because the full length B.t.k. HD-1 protein (~ 131 kD) … would be expected to be rapidly converted to the trypsin-resistant core protein (~ 63 kD) upon ingestion … the trypsin-resistant core of the B.t.k. HD-1 protein was considered an appropriate test material to assess the full length B.t.k. HD-1 protein."

Two studies were presented. One study compares the B.t.k. HD-1 CryIA(b) from the commercial microbial

Table 4. Summary of gene products in the modified plant

Gene product	*Breakdown products, byproducts and metabolic pathways*	*Expression*	*Activity of the gene product in the plant*	*Activity of the geneproduct in the environment*
CryIA(b) delta endotoxin protein	Tryptic peptide is active ingredient	Constitutive	Does not affect other metabolic pathways	Rapidly degraded by digestion (non lepidopteran) and in soil

product DIPEL with leaf tissue samples from the plant expressed in line 754-10-1. Line 754-10-1 was produced with the same transformation plasmids as MON 810, but has higher expression of the protein and therefore it was possible to purify a greater quantity of the protein for equivalence studies. The study demonstrated that the B.t.k. HD-1 trypsin resistant core from corn and *E. coli* are equivalent in molecular weight and immunological reactivity. Both DIPEL and line 754-10-1 contain a full length B.t.k. protein band at approximately 134 kD and the same trypsin resistant core of approximately 63 kD. Western blots demonstrated that the B.t.k. HD-1 core from line 754-10-1 and MON 810 were equivalent, therefore it is concluded that the protein produced by the *E. coli* is an appropriate substitute for the protein in MON 810.

Multiple protein products occur in the plant extract, in the commercial microbial product DIPEL and in the full-length protein preparation used in the acute toxicity study. A question about other fragments in the Western blots that are reactive to the CryIA(b) antibody probes and the meaning were addressed with the following. There should be no concerns since the acute oral toxicity study would have included these fragments. Any fragments outside the trypsin resistant core 28-610 amino acids (1-28 and 611-1150) possibly present in corn tissues show no amino acid homology with known toxins or allergens. Comparison of the CryIA(b) full length protein sequence against the same sequence data base indicates there is no homology with known toxins or allergens. Digestive fate shows that the protein is rapidly digested and the commercial microbial product DIPEL contains many fragments as well.

Western blots of proteins after treatment with trypsin show equivalent bands and that the 63 kD core is in both samples. MON 810 produces a protein product whose trypsin resistant core is equivalent to the trypsin resistant core of the B.t.k. 754-10-1 protein in terms of size and activity.

In a newer test than the one for 754-10-1, the equivalency was established directly between the bacterially and plant produced proteins in MON 810 using Western blot analysis, which was, "highly sensitive, specific for B.t.k. proteins and allows for comparison of the apparent molecular weights of proteins possessing immunological cross-reactivity in complex mixtures."

Leaf extracts of several IP lines and control lines were digested in trypsin to produce their B.t.k. HD-1 trypsin-resistant core protein and compared against the 63 kD *E. coli* produced trypsin- resistant core protein and the reference corn line MON 801 protein. The corn lines included MON 810 and its counterpart MON 818.

The Western blot analysis (Figure 6) shows a prominent band at the same molecular weight for MON 810 as the bacterial reference material. Smaller bands are also present and are assumed to be other B.t.k. HD-1 fragments. A band at 20 kD was seen in all extracts (both IP and control lines) and presumably represents a background non-specific cross-reactivity unrelated to the B.t.k. HD-1 protein.

"The results obtained in this study clearly establish that the B.t.k. HD-1 protein (as the trypsin-resistant core) produced by both *E. coli* and the IP corn lines analyzed in this study are equivalent. … the equivalence established … serves as the justification for using the safety data generated with the *E. coli*- produced (lot #I92017) protein to support the safety of the B.t.k. HD-1 protein expressed in these new insect protected corn lines."

Expression

Samples of field-grown IP corn (MON 810) and a control (MON 818) collected from US field sites were used to assess the expression level of CryIA(b), CP4 EPSPS, GOX and NPTII proteins. The control lines (MON818 and 819) are not genetically modified, but have "background genetics representative of the test substances." MON 818 is the counterpart for MON 810.

Leaf and grain samples were collected from six field sites distributed across the US corn growing regions, representative of the conditions where IP corn could be grown as a commercial product (2 in Illinois, 2 in Iowa, 1 each in Indiana and Nebraska). Whole plant and pollen samples were collected once from a single site (in Illinois). Over season leaf samples (taken every two weeks) were also collected from the Illinois site. Except for the pollen samples, B.t.k. HD-1, CP4 EPSPS and GOX protein levels were assessed using validated ELISAs specific for each protein. For the pollen samples, ELISA was used for the B.t.k. levels and Western blot analysis for CP4 EPSPS and GOX proteins.

Expression levels of the *cryIA(b)* gene were low in corn leaf, seed, pollen and whole plant tissues (Table 5). CP4 EPSPS, GOX and NPTII proteins were not detected. Average protein expression evaluated at six locations was 9.35 μg/g (f.w.) in leaves and 0.31 μg/g (f.w.) in seeds. Protein expression evaluated at one site was 4.15 μg/g (f.w.) in the whole plant and 0.09 μg/g (f.w.) in pollen, as determined from a single sample. Protein expression ranged from 7.93 to 10.34 μg/g (f.w.) in leaves, from 0.19 to 0.39 μg/g (f.w.) in grain and from

Table 5. Summary of levels of protein expression in MON 810 tissues[1]

Tissue	*Mean*	*Standard deviation*	*Range*
B.t.k. HD-1			
Leaf	9.35	1.03	7.93-10.34
Over season leaf[2]	9.78, 8.43, 4.91		
Pollen	0.09		
Whole plant[3]	4.15	0.71	3.65-4.65
Grain	0.31	0.09	0.19-0.39
CP4 EPSPS			
Leaf, over season leaf[2], whole plant, grain	nd	–	–
GOX			
Leaf, over season leaf[2], whole plant, grain	nd	–	–

[1] Unless indicated, values are in µg/g fwt (fresh weight). Unless indicated, the mean, standard deviation and range were over the six sites sampled. For those samples collected at one site see other notes.

[2] The numbers are means for the three separate sampling times collected at two week intervals.

[3] The mean and standard deviation were calculated from one site.

3.65 to 4.65 µg/g (f.w.) in the whole plant. Protein expression declined over the growing season as indicated by the Cry1A(b) levels present in leaves assayed over the growing season.

Tissue specificity, as stated by the company, was not expected since the *cryIA(b)* gene is "under the control of a CaMV promoter. Since this is a constitutive promoter that is not developmentally or tissue restricted, no specificity of expression to particular tissues is anticipated, although the CaMV promoter may be more or less active in certain cell types, as seen from the distribution of the CryIA(b) proteins in tissues." Neither were developmental stage specificity nor inducibility expected or found, because the CaMV promoter is a non-inducible constitutive promoter.

Western blot analysis of pollen (Figure 12) shows that the GOX gene is not expressed in MON 810 (lane 11).

For GM food assessments, expression in the consumed portion of the plant, in this case the grain, is the most important. The levels of expression in the grain of the novel protein range from 0.19 to 0.39 µg/g fresh weight.

The expression of the NPTII protein from the nptII gene, under the control of a bacterial specific promoter was tested for one of the lines used in this test (MON 801). The promoter was not active and, therefore, the gene does not express the protein in plant cells.

Breakdown products and metabolism

"The CryIA(b) protein does not have any specific breakdown products in plants. In the insect gut, the alkaline environment solubilizes the protein, which is then cleaved by proteases to yield the activated endotoxin. … As is commonly observed in Western blot analysis of Bt proteins, multiple polypeptides are apparent in extracts of plants expressing the *cryIA(b)* gene. These are recognized as breakdown products liberated as a result of protease action either in planta or during extraction."

Stability of the insert

MON 810 has been crossed into diverse corn genotypes for several generations and the efficacy of the line has been maintained. The molecular characterization of MON 810 was from the third generation of backcrossing and therefore the single insert appears to be stably integrated. Segregation data (Table 6) support a single active insert of the *cryIA(b)* gene segregating according to Mendelian genetics.

The *cryIA(b)* gene is stable through seven generations of crosses to one recurrent parent (B73) and six generations of crosses to a second, unrelated inbred (Mo17) (Table 7). The Chi square tests for the backcross to B73 and Mo17 did not deviate from expectations.

Assessment of possible toxicity

Introduction

Most of the studies were done using the insecticidally active trypsin-resistant core *E. coli* produced protein and not with plant-produced protein. The test proteins produced in *E. coli* are chemically and functionally the same as the plant-produced proteins (section 4.1.1).

Table 6. Segregation data of MON 810 progeny

Generation	*Description*	*Actual*	*Expected*	*ChiSq*
BC0F1[1]	Derived from cross of R0 with an inbred line	44:47	45.5:45.5	0.044*
BC1F1[2]	Derived from cross of BC0F1 plants to the same inbred line used to cross the R0 plant	10:4	7:7	1.786*
BC1F2 progeny[3]	Derived from cross of individual BC0F2 plants by a non-transgenic tested	69:181:77	81.75:163.5:81.75	4.138#

[1] *Expressed as number of expressing plants: number of non-expressing plants based on ECB feeding assay.*
[2] *Expressed as number of expressing plants: number of non-expressing plants based on CryIA(b) ELISA.*
[3] *Expressed as number of ear rows with homozygous number of expressing plants: number of ear rows with segregating plants: number of ear rows with homozygous susceptible plants based on ECB feeding assay.*
* *Not significant at p=0.05 (chi square = 3.94, 1df); # not significant at p=0.05 (chi square = 5.99, 2 df).*

Table 7. Stability of gene transfer based on segregation data for backcross derivatives of MON 810 with two unrelated inbred lines (B73 and Mo17)

Generation[1]	*Actual*	*Expected*	*Chi square*
BC6F1 (B73)	8:13	10.5:10.5	0.762*
BC5F1 (Mo17)	11:11	11:11	0.045*

[1] *Data expressed as number of expressing plants: number of non-expressing plants based on CryIA(b) ELISA.*
* *Not significant at p=0.05 (chi square = 3.84, 1 df).*

Some of the food safety considerations are based on CryIA(b) characterization and digestive fate studies in simulated gastric and intestinal fluids.

Protein specificity

The CryIA(b) protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH, however, is solubilized by the alkaline gut of larval insects. The solubilized protein is then activated by the proteases in the insect gut, which diffuses through the peritrophic membrane to the midgut epithelium, binding to specific high affinity receptors on the surface. This paralyzes the gut due to changes in electrolytes and pH causing the insect to stop feeding and die.

There are no similar receptors for the protein delta-endotoxins of Bt species on the surface of mammalian intestinal cells, therefore mammals are not susceptible to these proteins. Also, absence of adverse effects in humans is supported by numerous reviews on the safety of Bt proteins.

Comparison to toxin databases

The Cry1A(b) amino acid sequence was compared to known protein toxins. Similarity to a known toxin could trigger toxicological testing to address potential impact of the homology. B.t.k. HD-1 protein was compared to the amino acid sequences of 2632 toxins collected from public domain genetic databases (GenBank, EMBL, PIR and Swiss Prot) for homology. The results confirm that the B.t.k. HD-1 protein is homologous to Bt insecticidal crystal proteins, but no amino acid homology was detected for other protein toxins. The closest match is shown in Figure 14.

Mouse acute oral gavage

An acute oral toxicity study (7 days) was done with albino mice using *E. coli* produced protein (converted to the trypsin resistant core) and tested for purity, potency and stability. The protein was administered by gavage to mice at targeted doses of 0, 400, 1000 and 4000 mg/kg. The highest dose represents the maximum hazard dose concept outlined in US Subdivision M Guidelines for biochemical pesticides. One group was dosed with 4000 mg/kg of bovine serum albumin (BSA) as a protein control.

No treatment related adverse effects were observed (Table 8) and no statistical differences in body weight measures or food consumption were seen. No differences were seen in gross pathology between the groups. The LC50 of the B.t.k HD-1 (truncated) protein in mice is greater than 4000 mg/kg with the NOEL set at that value.

Potential toxic contaminants

In response to queries about possible changes in contaminant levels due to the introduction of the

Table 8. Results of acute mouse gavage test with CryIA(b) protein

Test group	*Weight pretest (g)*	*Weight at end (g)*	*Food consumption (mean g/day)*
Vehicle control (buffer)	31.1 [25.5]	30.8 [25.1]	5.3 [6.4]
Control (BSA 4000*)	31.1 [25.4]	31.0 [24.7]	6.2 [7.3]
400 Bt protein	31.1 [25.4]	30.5 [25.2]	5.3 [8.0]
1000 Bt protein	31.0 [25.3]	31.1 [25.0]	5.3 [8.0]
4000 Bt protein	31.0 [25.5]	30.5 [25.5]	5.5 [8.0/7.4]

*[females] / *mg/kg body weight*

Table 9. Dissipation of B.t.k. HD-1 protein insecticide activity in simulated gastric fluids

B.t.k. *HD-1*	*Tobacco budworm mortality*		*% change*
(µG/nL)	*0*	*2 minutes*	
0.75	29	3	-90
7.5	69	8	-88
75	94	24	-74

Table 10. Dissipation of B.t.k. HD-1 protein insecticide activity in simulated intestinal fluids

B.t.k. *HD-1*	*Tobacco budworm mortality*		*% change*
(µG/nL)	*0*	*19,5 hours*	
0.75	26	25	-4
7.5	76	61	-20
75	100	90	-10

cryIA(b) gene, the company notes that for alflatoxins, tests with MON 810 from the 1993 field trial did not detect alflatoxins and therefore the test was not repeated.

DIMBOA (2,4-dihodroxy-7-methoxy-1,4-bezoxanin-3-one) is not present in seeds of cereals and therefore does not pose a hazard to consumers of grain products.

Metabolic degradation in simulated gastric and intestinal fluids

Purified CryIA(b) protein (B.t.k HD-1 as expressed in *E. coli*) degrades rapidly in vitro using simulated digestive fluids. In the simulated gastric fluid, more than 90% of the protein degraded within two minutes, as detected by Western blot analysis (Figure 15). Lanes 6-11 are incubations at 0, 10, 20, 30, 60 and 120 seconds. Protein bioactivity detected using an insect bioassay also dissipated quickly with 74-90% of the added protein dissipated within two minutes (Table 9), the earliest time point measured. In a human stomach, approximately 50% of solid food empties to the intestines in two hours and liquids in about 25 minutes.

In the simulated intestinal fluid, the purified Cry1A (b) protein did not degrade substantially after 19.5 hours as assessed by Western blot (Figure 16, lanes 8-11 are incubations at 0, 60 minutes, 4 hours and 19.5 hours) and insect assay (Table 10). This was anticipated since the tryptic core of Bt insecticidal proteins is known to be relatively resistant to serine proteases like trypsin, a key protease in intestinal fluid. The insect used for the insect assay studies was the tobacco budworm.

Assessment of possible allergenicity

Humans consume large quantities of proteins daily and allergenic reactions are rare. One factor to consider is whether the source of the gene being introduced into the plants is known to be allergenic. Bt does not have a history of causing allergy. “In over 30 years of commercial use, there have been no reports of allergenicity to Bt, including occupational allergies associated with manufacture of products containing Bt.” Further, protein allergens need to be stable in peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response. Tests above show that the CryIA(b) protein does not survive under simulated gastric digestion. Another common factor of allergenic proteins is that they occur in high levels in the foods (*e.g.*, allergens in milk, soybean, peanuts). This is not the case with the CryIA(b) protein which is present at approximately 0.19-0.39 µg/g fresh weight of corn seed.

The company stated that Comparing sequences of amino acids to known allergens and gliadins is a useful first approximation of potential allergenicity or association with coeliac disease. A database of 219 protein sequences associated with allergy and coeliac disease assembled from genetic databases (GenBank, EMBL, PIR and Swiss Prot) was searched for sequences similar to B.t.k. HD-1 protein. “Most major … food allergens have been reported and the important IgE

binding epitopes of many allergenic proteins have been mapped. The optimal peptide length for binding is between 8 and 12 amino acids. T-cell epitopes of allergenic proteins and peptide fragments appear to be at least 8 amino acids in length. Exact conservation of epitope sequences is observed in homologous allergens of disparate species. … an immunologically relevant sequence comparison test for similarity … is defined as a match of at least eight contiguous identical amino acids." No biologically significant homology nor immunological significant sequence similarities were found. The best match is shown in Figure 17. The results establish that B.t.k. HD-1 protein shares no significant similarity with known allergen or gliadin proteins.

In summary, the low levels of the protein in the corn, combined with the digestive lability and the lack of homology with known allergenic sequences indicate that this protein does not possess allergenic properties. Coupled with the history of use as a microbial control agent with no allergenic concerns, this indicates that there is no reason to believe that CryIA(b) should pose any significant allergenic risks for the consumption of products produced from insect-protected corn.

Compositional analyses of key Components, evaluation of metabolites, food processing and nutritional modification

Introduction

Nutritional data are important relative to dietary exposure to corn products. While little whole kernel or processed corn is directly consumed by humans, corn based food ingredients such as starch and corn oil are used.

Compositional data

Samples for composition analysis were collected at the same time and from the same six sites used for analysis of expression levels in corn grain for a one-time experiment.

Corn seed (grain) samples of MON 810 and the control MON 818 were analyzed for the following components and compared with available literature values:

- Proximates (moisture, protein, ash, fat, crude fibre)*
- Calories
- Carbohydrate
- Starch
- Fatty acid profile*
- Sugar profile
- Amino acid composition*
- Tocopherols*
- Phytic acid*
- Minerals (calcium, phosphorus)* as summarized in Table 11.

Parameters with an asterisk (*) are considered for feed assessments, while the other parameters (often derived from calculations) are not commonly considered.

Carbohydrates were not measured but deduced using the following calculation: % carbohydrates = 100% - (% protein + % fat + % ash + % moisture). Also, calories was a derived parameter using the following USDA approved calculation: calories (kcal/100g) = (4 * % protein) + (9 * % fat) = (4 * % carbohydrates).

There were no significant differences for the variables protein, fat, ash, carbohydrates, calories and moisture between the IP corn and its control and both were within the reported values from the literature.

MON 810 contained eight amino acids (cystine, tryptophan, histidine, phenylalanine, alanine, proline, serine and tyrosine), which were statistically different from the control. The mean values for six of these (all except cystine and histidine) are within literature ranges. Cystine and histidine for both lines were statistically higher than the literature range but within the range (1.9-2.3%) observed for two (MON 800/801) similar lines. The level of histidine for MON 810 (3.1%) is within the range of another previous study for two lines of similar genetic backgrounds.

For fatty acids and carbohydrates measured (starch, fructose, glucose, sucrose and phytic acid), no significant differences were found between the control and the IP lines. Crude fiber values in MON 810 grain (2.6%) were statistically different from MON 818, but both values were within the literature range (2.0-5.5%).

Tocopherols are naturally present in corn oil and have vitamin E potency. The gamma tocopherol is one-tenth as active as the alpha and is therefore not considered an important component of the corn grain. MON 810 values for the alpha and gamma tocopherols were statistically similar to the control but the beta tocopherol differs statistically from the control (Table 11).

For the minerals calcium and phosphorus, calcium levels in MON 810 were statistically higher than for MON 818, but within ranges reported for tests with MON 800/801. No statistical differences were found for phosphorus.

Table 11. Comparison of compositional analysis for MON 810 corn grain with control (MON 818) and literature values

Component	*MON 810[1] mean (range)[2]*	*MON 818 mean (range)[2]*	*Literature value[4] mean (range) [MON 800/801 range]*
Proximate analysis			
Protein[3]	13.1 (12.7-13.6)	12.8 (11.7-13.6)	9.5 (6.0-12.0) 12.3 (9.7-16.1) [11.2-13.6]
Fat	3.0 (2.6-3.3)	2.9 (2.6-3.2)	4.3 (3.1-5.7), 4.6 (2.9-6.1) [3.8-4.2]
Ash[3]	1.6 (1.5-1.7)	1.5 (1.5-1.6)	1.4 (1.1-3.9) [1.5-1.8]
Carbohydrate[3]	82.4 (81.8-82.9)	82.7 (81.7-83.8)	not reported [80.8-83.0]
Calories/100g	408.4 (407.0-410.1)	408.5 (406.0-410.1)	not reported [412.6-415.7]
Moisture %	12.4 (11.0-14.4)	12.0 (10.6-14.2)	16.0 (7-23) [13.0-15.8]
Amino acid composition - nutritionally essential[5]			
Methionine	1.7 (1.6-1.9)	1.7 (1.6-1.7)	1.0-2.1 [2.0-2.6]
Cystine	2.0* (1.9-2.1)	1.9 (1.8-2.0)	1.2-1.6 [1.9-2.3]
Lysine	2.8 (2.5-2.9)	2.8 (2.7-2.9)	2.0-3.8 [2.6-3.4]
Tryptophan	0.6* (0.5-0.7)	0.6 (0.4-0.6)	0.5-1.2 [0.5-0.6]
Threonine	3.9 (3.7-4.4)	3.8 (3.7-3.9)	2.9-3.9 [3.9-4.2]
Isoleucine	3.7 (3.3-4.1)	3.8 (3.6-4.0)	2.6-4.0 [3.5-3.8]
Histidine	3.1* (2.9-3.3)	2.9 (2.8-3.0)	2.0-2.8 [2.8-3.3]
Valine	4.5 (4.1-4.9)	4.6 (4.3-4.8)	2.1-5.2 [4.2-4.8]
Leucine	15.0 (14.1-16.7)	14.5 (13.8-15.0)	7.8-15.2 [13.6-14.5]
Arginine	4.5 (4.2-4.7)	4.5 (4.2-4.7)	2.9-5.9 [4.1-5.0]
Phenyalanine	5.6* (5.2-5.6)	5.4 (5.2-5.6)	2.9-5.7 [5.2-5.6]
Glycine	3.7 (3.4-4.0)	3.7 (3.5-3.8)	2.6-4.7 [3.4-4.2]
Amino acids - nonessential[5]			
Alanine	8.2* (7.8-8.9)	7.8 (7.5-8.0)	6.4-8.0 [7.8-8.2]
Aspartic acid	7.1 (6.4-8.2)	6.6 (6.3-6.8)	5.8-7.2 [6.7-7.3]
Glutamic acid	21.9 (20.4-24.4)	21.1 (201.-21.6)	12.4-19.6 [19.9-21.4]
Proline	9.9* (9.7-10.5)	9.6 (9.4-9.8)	6.6-10.3 [9.0-9.4]
Serine	5.5* (5.3-5.9)	5.2 (5.1-5.4)	4.2-5.5 [5.5-6.1]
Tyrosine	4.4* (4.1-4.8)	4.0 (3.9-4.1)	2.9-4.7 [3.8-4.3]
Fatty acids[6]			
Palmitic (16:0)	10.5 (10.2-11.1)	10.5 (10.2-10.7)	7-19 [10.2-10.9]
Stearic (18:0)	1.9 (1.7-2.1)	1.8 (1.8-1.9)	1-3 [1.6-3.1]
Oleic (18:1)	23.2 (21.5-25.4)	22.8 (21.6-23.9)	20-46 [21.2-25.9]
Linoleic (18:2)	62.6 (59.5-64.7)	63.0 (61.8-64.6)	35-70 [58.9-65.0]
Linolenic (18:3)	0.8 (0.7-0.9)	0.9 (0.8-0.9)	0.8-2 [0.9-1.1]
Carbohydrates and fiber7			
Starch %	67.6 (65.3-69.7)	66.9 (64.6-69.0)	64-78.0 [63.7-71.5]
Crude fiber %	2.6* (2.5-2.8)	2.4 (2.3-2.5)	2.0-5.5 [1.98-2.61]
Sugars[8]			
Fructose	0.32 (0.23-0.35)	0.27 (0.22-0.40)	[0.47-0.96]
Glucose	0.44 (0.34-0.47)*	0.93 (0.79-1.12)	[0.47-1.03]
Sucrose	0.93 (0.79-1.12)	0.93 (0.68-1.11)	[0.40-0.94]
Phytic acid %	0.86 (0.81-0.91)	0.84 (0.79-0.91)	0.7-1.0 [0.45-0.57]
Tocopherols (mg/kg)			
Alpha	10.4 (9.7-11.3)	10.9 (9.9-12.1)	3.0-12.1 [7.3-12.3]
Beta	8.5* (8.1-9.2)	7.5 (7.0-7.9)	[7.9-10.7]
Gamma	20.2 (15.3-24.8)	21.6 (18.8-27.8)	[21.7-42.5]

(Continued)

Table 11. (cont.)

Component	*MON 810[1] mean (range)[2]*	*MON 818 mean (range)[2]*	*Literature value[4] mean (range) [MON 800/801 range]*
Inorganic components[7]			
Calcium %	0.0036* (0.0033-0.0039)	0.0033 (0.0029-0.0037)	0.01-0.1 [0.003-0.004]
Phosphorus %	0.358 (0.334-0.377)	0.348 (0.327-0.363)	0.26-0.75 [0.311-0.368]

*1 Values with * are statistically different from MON 818.*
2 Values reported are means of six samples from six sites. Ranges are the highest and lowest values across those sites.
3 Percent dry weight of samples.
4 Where there are more than one value, this indicates more than one published source.
5 Values for amino acids reported as percent of total protein.
6 Values for fatty acids are % total lipid. Other fatty acids were below the limit of detection of the assay.
7 Values on a dry weight basis.
8 Sugars measured as g/100g. Galactose, lactose and maltose were also measured, but values were below the limit of detection.

The company concluded, "Based on these data, it was concluded that there are no meaningful compositional differences between the IP corn lines ... and the control line, MON 818."

Additionally, the company summarized its Nutritional analysis conclusions, "nutritional composition … falls within the ranges of each nutrient measures for non-modified corn lines. It can be concluded that there appears to be no meaningful effect on corn plant nutrient levels. Phenotype was not affected in any of the numerous ways that were measured. Of the vitamins and minerals measured there were no practical differences reported. In terms of nutritional composition, MON 810 may be considered to be substantially equivalent to regular corn." ●

Case study 2

Safety assessment of genetically modified high oleic acid soybeans

Preface

The sale of food derived from high oleic acid soybean lines G94-1, G94-19 and G168 (Application A387) was approved in Australia and New Zealand in November 2000, following completion of a comprehensive safety assessment. Food Standards Australia New Zealand (FSANZ) conducts the safety assessments of genetically modified foods based upon internationally accepted principles for establishing the safety of foods derived from GM plants.

The findings of the FSANZ safety assessment were published as the "Final Risk Analysis Report: Application A387 - Food derived from high oleic soybean lines G94-1, G94-19, and G168".

Parts of the data and information on high oleic acid soybeans provided to FSANZ for assessment have been summarised into this case study for training purposes.

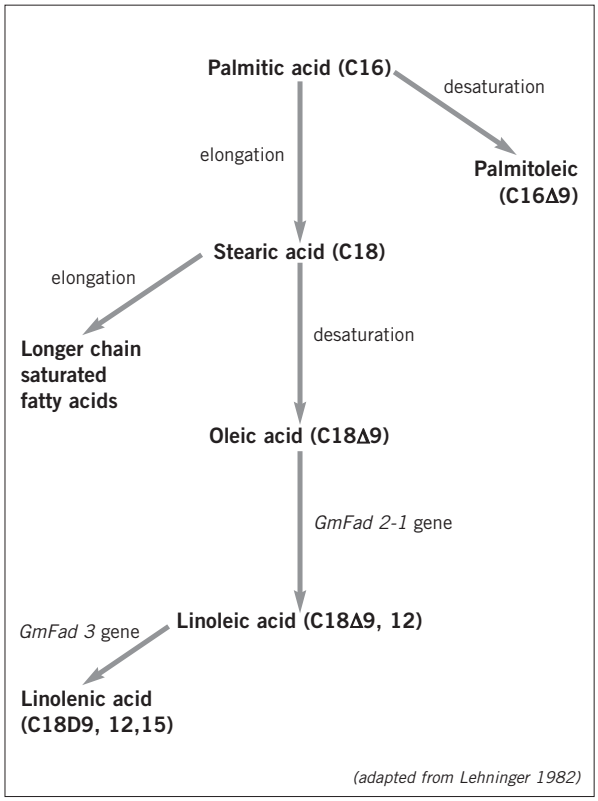
Disclaimer

In order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original application. Certain information has been reduced to summaries and the present data as presented in the case study are only a subset of that actually submitted. The case study in no way constitutes a complete application not is it to be considered a complete safety assessment. To that end, the use of this information in the form of a training tool does not constitute an endorsement of the information or product nor should it be considered a reflection of the original submission.

Description of the recombinant-DNA plant

Optimum Quality Grains LLC (a joint venture between DuPont and Pioneer Hi-Bred International, Inc) originally intended to develop soybeans with two introduced traits: (a) increased lysine in the meal fraction and (b) increased oleic acid, a monounsaturated fatty acid, in the oil fraction. However, during development, it was decided not to pursue the high-lysine trait. The new variety therefore has been genetically modified only to contain increased levels of oleic acid. The soybeans are referred to as high oleic acid soybeans.

The high oleic acid trait was generated by the transfer of a second copy of a soybean fatty acid desaturase gene (GmFad 2-1) to a high yielding commercial variety of soybean. The fatty acid desaturase is responsible for the synthesis of linoleic acid, which is the major polyunsaturated fatty acid present in soybean oil. The presence of a second copy of the fatty acid desaturase gene causes a phenomenon known as "gene silencing" which results in both copies of the fatty acid desaturase gene being "switched off", thus preventing linoleic acid from being synthesised and leading to the accumulation of oleic acid in the developing soybean seed. The pathway for the synthesis of long chain fatty acids in plants is depicted below.



Soybean oil has poor oxidative stability due to naturally high levels of polyunsaturated fatty acids (such as linoleic acid). High oleic acid soybean oil is considered to have superior properties to that of standard soybean oil because of its reduced levels of the oxidatively unstable polyunsaturated fatty acids. This means that high oleic acid soybean oil may be used for a number of food applications, including deep fat frying, without the need for additional processing, such as chemical hydrogenation. High oleic acid soybean oil is also considered to offer improved nutritional properties compared to conventional soybean oil or partially hydrogenated soybean oil because of the increased levels of monounsaturated fatty acids.

Oil from high oleic soybeans is intended to be used predominantly for spraying and frying applications in the

food industry and food services and might replace heat stable fats and oils such as hydrogenated soybean and rapeseed oil or palm oil/vegetable oil blends.

Description of the host plant and its use as food

Soybeans (*Glycine max*) are grown as a commercial crop in over 35 countries worldwide and have a long history of safe use as both human food and stockfeed. The major producers of soybeans are the United States, Argentina, Brazil and China, accounting for 90% of world production.

There are three major soybean commodity products: seeds, oil and meal. There is only limited feed use, and no food use, for unprocessed soybeans, as they contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors, making them unsuitable for human consumption. Appropriate heat processing inactivates these compounds.

Whole soybeans are used to produce soy sprouts, baked soybeans, and roasted soybeans. The soybean hulls can be processed to create full fat soy flour and the traditional soy foods such as miso, tofu, soymilk and soy sauce.

Before processing, soybeans are graded, cleaned, dried and de-hulled. The soybean hulls are further processed to create fibre additives for breads, cereals and snacks and are also used for stockfeed. After de-hulling, soybeans are rolled into full fat flakes that may be either used in stockfeed or processed further into full fat flour. Crude soybean oil is then extracted from the flakes by immersing them in a solvent bath. Crude lecithin is then separated from the oil, which is further refined to produce cooking oil, margarine and shortening. After the oil is extracted from the flakes, the solvent is removed and the flakes are dried for use in the production of soy flour, soy concentrates and soy isolates. De-fatted soy flakes are also used in stockfeed.

Finished food products containing soybean ingredients therefore include beer, noodles, breads, flours, sausage casings, pastries, crackers, meat substitutes, milk substitutes and confectionery among other things.

The elite soybean cultivar A2396, which has been used as the host for the high oleic acid trait described in this application, is an Asgrow Seed Company early Group II maturity soybean variety that has high yield potential. Protein and oil characteristics are said to be similar to other soybeans at 40% protein and 22% oil on a dry weight basis.

Description of the genetic modification

Methods used in the genetic modification

Plasmid DNA carrying the genes of interest, was introduced into meristem tissue of elite soybean line A2396 by microprojectile bombardment, or biolistic transformation. The bombarded cells are incubated on a tissue culture medium, which supports callus growth. The cells that have taken up the DNA were selected by picking those that express an introduced marker gene, GUS (a fluorescent marker protein).

Novel genes

The *GmFad 2-1* gene

In soybean, there are two Fad 2 genes, but only the *GmFad 2-1* gene is expressed in the developing seed (Heppard *et al.*, 1996). The expression of *GmFad 2-1* increases during the period of oil deposition, starting around 19 days after flowering, and its gene product is responsible for the synthesis of the polyunsaturated fatty acids found in the oil fraction. The second Fad 2 gene (*GmFad 2-2*) is expressed in the seed, leaf, root and stem at a constant level and its gene product is responsible for the synthesis of the polyunsaturated fatty acids present in cell membranes.

The presence of a second copy of the *GmFad 2-1* gene in the soybean causes a phenomenon known as "gene silencing" which results in both copies of the *GmFad 2-1* gene (the transferred copy as well as the original soybean copy) being "switched off", thus preventing linoleic acid from being synthesised and leading to the accumulation of oleic acid in the developing soybean seed.

Gene silencing in plants can occur at both transcriptional (TGS) and post-transcriptional (PTGS) levels. The primary mechanism of TGS is thought to be methylation of the promoter sequences. Methylation of promoters is thought to block their interaction with transcription factors or alter the chromatin structure of the DNA thus suppressing transcription, however these mechanisms remain unclear (Wang and Waterhouse, 2001). PTGS was initially referred to as 'co-suppression' because in experiments involving the transformation of petunia with a sense chalcone synthase transgene the expression of both the transgene and the corresponding endogenous gene was suppressed. PTGS involves the

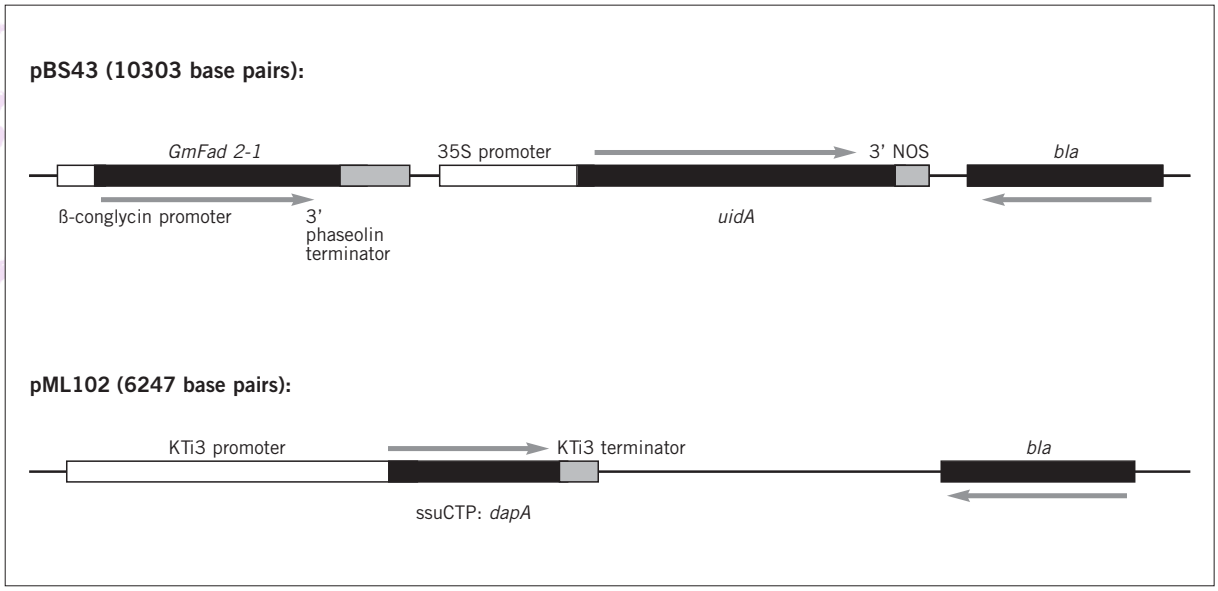


Table 1: Description of the gene expression cassettes in pBS43 and pML102

Cassette	*Genetic element*	*Source*	*Function*
GmFad 2-1 expression cassette (pBS43)	ß-conglycinin promoter	α^1-subunit of β-conglycinin seed storage protein of soybean (Barker *et al.* 1988)	Seed specific promoter that allows high level gene expression during seed development
	GmFad 2-1 coding region	Protein coding sequence of the δ-12 fatty acid desaturase from soybean (Okuley *et al.* 1994, Heppard *et al.* 1996)	The endogenous enzyme adds a second double bond to oleic acid thus converting it to linoleic acid
	phaseolin 3' terminator	The 3' terminator region from the phaseolin seed storage protein of green bean *Phaseolis vulgaris* (Doyle *et al.* 1986)	Contains signals for termination of transcription and directs polyadenylation
GUS expression cassette (pBS43)	35S promoter	A promoter derived from the cauliflower mosaic virus (CaMV) (Odell *et al.* 1985)	Promoter of high level constitutive gene expression in plant tissues
	Cab 22L non-translated leader	The 5' untranslated leader from the photosynthetic *22L* chlorophyll a/b binding protein (Cab22L) promoter of *Petunia hybrida* var. Mitchell (Harpster *et al.* 1988)	The untranslated leader sequence helps to stabilise mRNA and improve translation
	uidA coding region	Protein coding sequence of the enzyme β-glucuronidase (*uidA* gene) from *Escherichia coli* (Jefferson *et al.* 1985)	Colourimetric marker used for selection of transformed plant lines
	NOS 3'	The 3' terminator region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Depicker *et al.* 1982, Bevan *et al.* 1983)	Contains signals for termination of transcription and directs polyadenylation
dapA expression cassette (pML102)	Kti3 promoter	Promoter from Kunitz trypsin inhibitor gene 3 of soybean (Jofuki and Goldberg 1989).	Seed specific promoter that allows high level gene expression during seed development.
	ssu CTP	The N-terminal chloroplast transit peptide sequence from the soybean small subunit of Rubisco (Berry-Lowe *et al.* 1982)	Directs the protein into the chloroplast which is the site of lysine biosynthesis
	dapA coding region	Coding sequence of the *Corynebacterium dapA* gene encoding the lysine insensitive version of the enzyme dihydrodipicolinic acid synthase (DHDPS) (Bonnassie *et al.* 1990, Yeh *et al.* 1988)	Expression of *Corynebacterium* DHDPS deregulates the lysine biosynthetic pathway resulting in accumulation of free lysine (Falco *et al.* 1995)
	Kti3 3' terminator	The 3' terminator region from Kunitz trypsin inhibitor gene 3 from soybean (Jofuki and Goldberg 1989)	Contains signals for termination of transcription and directs polyadenylation

Table 2: Description of other genetic elements transferred to high oleic acid soybeans

Cassette	*Genetic element* *Source*	*Function*
lac	An incomplete copy of the *lac* operon which contains a partial *lacI* coding sequence, the promoter P_{lac}, and a partial coding sequence for β-D-galactosidase (*lacZa'*)	These genes are not intact and no longer function in *E. coli*
ori	Origin of replication from the high copy number *E. coli* plasmid pUC19	Allows plasmids to replicate in *E. coli*
bla	Gene coding for the enzyme β-lactamase from *E. coli*	Confers ampicillin resistance to *E. coli*
f1 ori	Bacteriophage f1 origin of replication.	Origin of replication recognised by bacteriophage f1 to produce single stranded DNA. The f1 origin is not recognised unless a phage f1 is present

failure to accumulate messenger RNA in the cytoplasm and thus no expression products are produced. It is now widely accepted that double stranded RNA can cause PTGS in plants through a process that involves sequence-specific RNA degradation (Voinnet, 2002).

The *dapA* gene

The *dapA* gene codes for the enzyme dihydrodipicolinic acid synthase (DHDPS), which is responsible for catalysing the first step in the metabolic pathway for the synthesis of the essential amino acid lysine (Brock *et al.*, 1984). The DHDPS found in plants is inhibited by lysine, whereas the *dapA* gene transferred to the soybeans, which was derived from *Corynebacterium*, codes for a form of DHDPS that is insensitive to inhibition by lysine. In previous experiments it has been shown that expression of the lysine-insensitive DHDPS, encoded by the *Corynebacterium dapA* gene, will result in more than a 100-fold increase in the accumulation of free lysine in the seeds, essentially doubling total seed lysine content (Falco *et al.*, 1995).

The objective of transforming soybean with both the soybean *GmFad 2-1* gene and the *Corynebacterium dapA* gene was to produce transgenic soybeans with increased lysine in their meal fraction, due to expression of the lysine insensitive form of DHDPS, and a reduced level of polyunsaturated fatty acids in their oil fraction, due to silencing of the *GmFad 2-1* gene (described above).

uidA gene

In addition to the primary genes, the soybeans also contain a visual marker gene, the *uidA* gene from *Escherichia coli* (Jefferson *et al.*, 1985). The protein product of this gene, β-glucuronidase (GUS), is an enzyme that can be used to catalyse a colourimetric reaction resulting in the production of a blue colour in transformed plant tissues.

Gene constructs

Two circular plasmids were used in the transformation, pBS43 and pML102, containing the three gene expression cassettes, one for each gene of interest, *GmFad 2-1* and *dapA*, and one for the reporter gene, *uidA*. Both plasmids pBS43 and pML102 also contained the antibiotic resistance marker gene, *bla*. The plasmids are shown in the diagram (Fig. 1) in linear form, with the novel genes in black. Table 1 contains a description of each gene and its regulatory elements.

Other genetic elements

In addition to the gene expression cassettes described in Table 1 above, a number of other genetic elements, including the antibiotic resistance marker gene, were also present in the plasmid DNA. These genetic elements are described in Table 2.

These genetic elements are present in most *E. coli* cloning vectors and are well described (Sambrook *et al.*, 1981). They are used to assist in the manipulation of DNA sequences as well as direct gene expression in *E. coli*.

Characterisation of the genetic modification

Selection of plant lines

The method used in the transformation did not necessarily result in the successful transfer of both plasmids to the soybeans, therefore a large number of transformed plants needed to be screened to identify those with the two traits of interest.

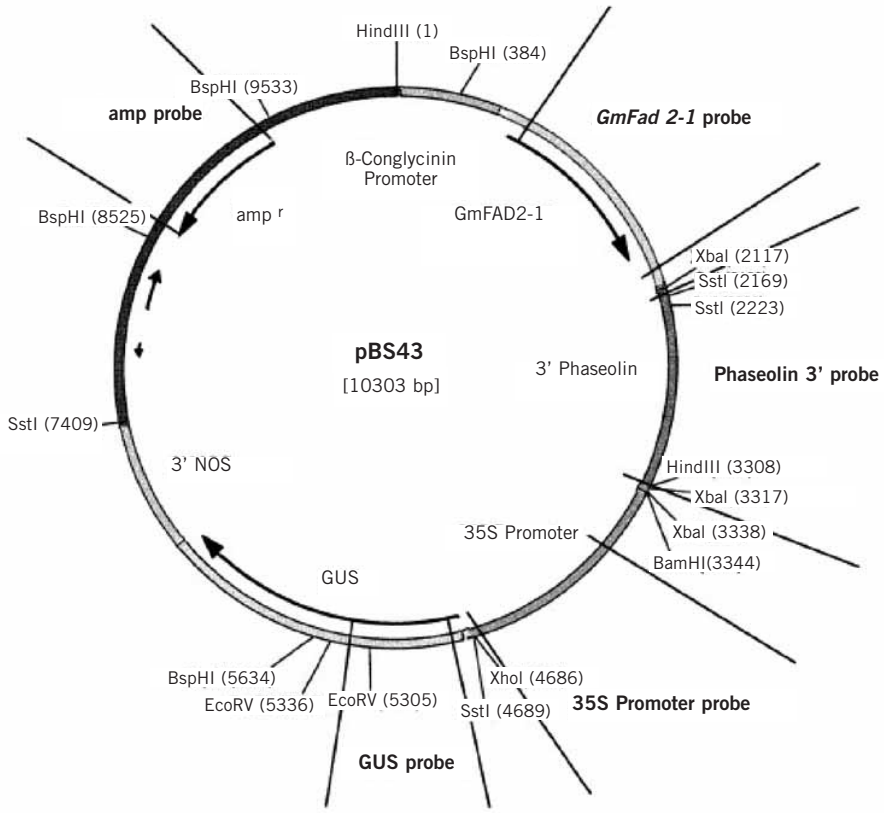


Figure 1: Plasmid map of pBS43. Figure indicates the location of hybridisation probes and restrictions enzyme sites used for Southern blot analysis of high oleic soybeans.

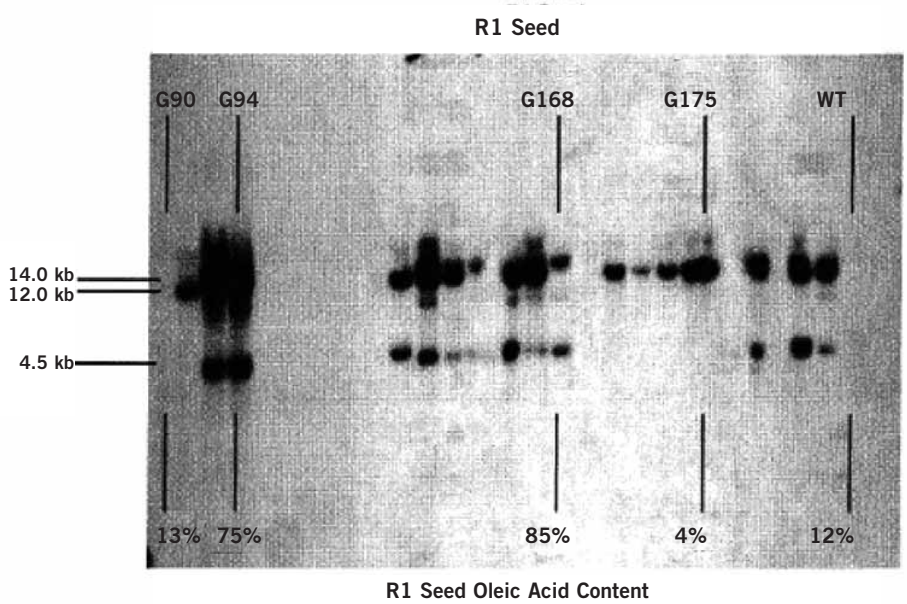


Figure2. Southern blot of DNA isolated from leaf tissue of event 260-05 R1 plants. Plants were grown from chipped seeds analysed for fatty acid composition. The genomic DNA was digested with *BamHI* and probed with the phaseolin 3' probe to detect the integration of the *GmFad 2-1* construct.

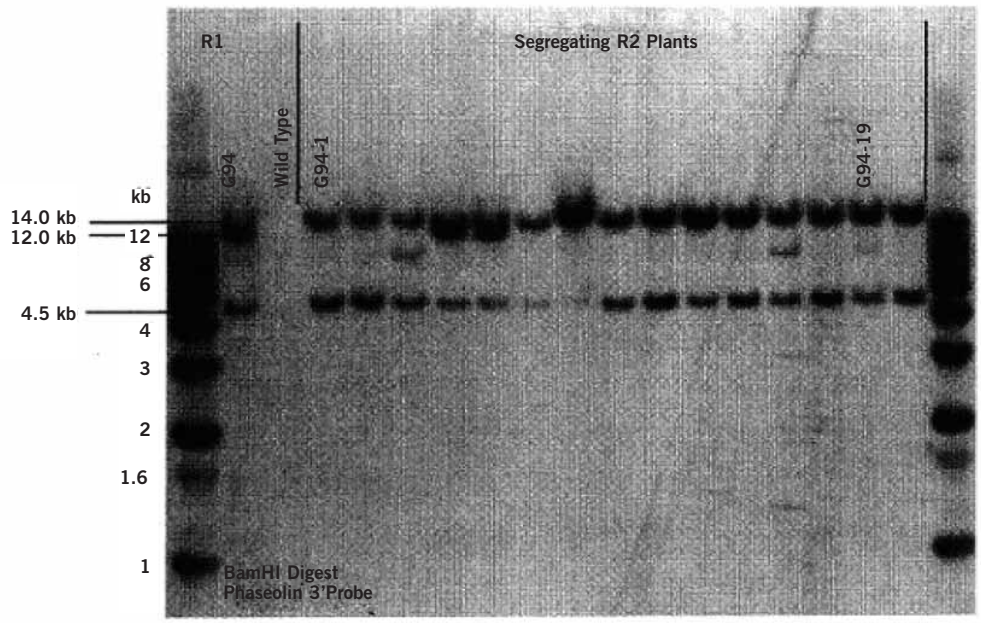


Figure 3. Southern blot on R1 and R2 leaf tissue from G94 R1 seed. The genomic DNA was digested with BamHI and probed with the phaseolin 3' probe to detect the integration of the GmFad 2-1 construct. The G94 seed has three different sized fragments of DNA that hybridise with the probe. G94-1 and G94-19 have only two – at 14.0 Kb and 4.5 Kb.

As the GUS reporter gene is linked to the *GmFad 2-1* gene, the population of transformed plants was first screened for GUS activity. The GUS-positive plants were then tested using the polymerase chain reaction (PCR), for the presence of the *GmFad 2-1* gene. From this initial screening one plant (event 260-05) was identified. Small samples were taken from the seeds of plant 260-05 (the R1 generation) and screened for fatty acid composition and lysine content. Four different fatty acid profiles in combination with lysine changes were identified among the R1 seeds:

1. Seeds with ≥80% oleic acid content and normal lysine levels (G168);
2. Seeds with about 72% oleic acid content and increased lysine levels (G94);
3. Seeds with about 4% oleic acid content and increased lysine levels (G175); and
4. Seeds with oleic acid and lysine levels similar to that of the untransformed line A2396 (G90).

Southern blot hybridisation was used to analyse genomic DNA from seeds from the four transformed lines described above. Southern blotting is a sensitive technique used to detect specific sequences within DNA fragments that have been separated according to size using gel electrophoresis (Southern, 1975). This provides information on the number of inserts of the T-DNA, and the number of insertion sites (i.e., the number of loci) in the genome of the soybean plants. It is also possible to some extent to determine whether the inserted T-DNA copies are whole (intact) or partial copies.

Genomic DNA was extracted from the seed samples, digested with the restriction enzyme BamHI and probed with the 3' region of the phaseolin terminator to detect the *GmFad 2-1* gene expression cassette. BamHI cuts once in the plasmid pBS43 and would be expected to result in one hybridizing band for each copy of the plasmid inserted into the genome. The map of pBS43 with restriction sites and locations of probes is shown in Figure 1. The results of the Southern blot are shown in Figure 2.

Three different banding patterns can be seen in Figure 2 The results for G168 show two hybridising bands of 14.0 Kb and 4.5 Kb, indicative of two *GmFad 2-1* genes. G175 has one band only, corresponding to 12.0 kb. All three hybridising fragments are present in G94.

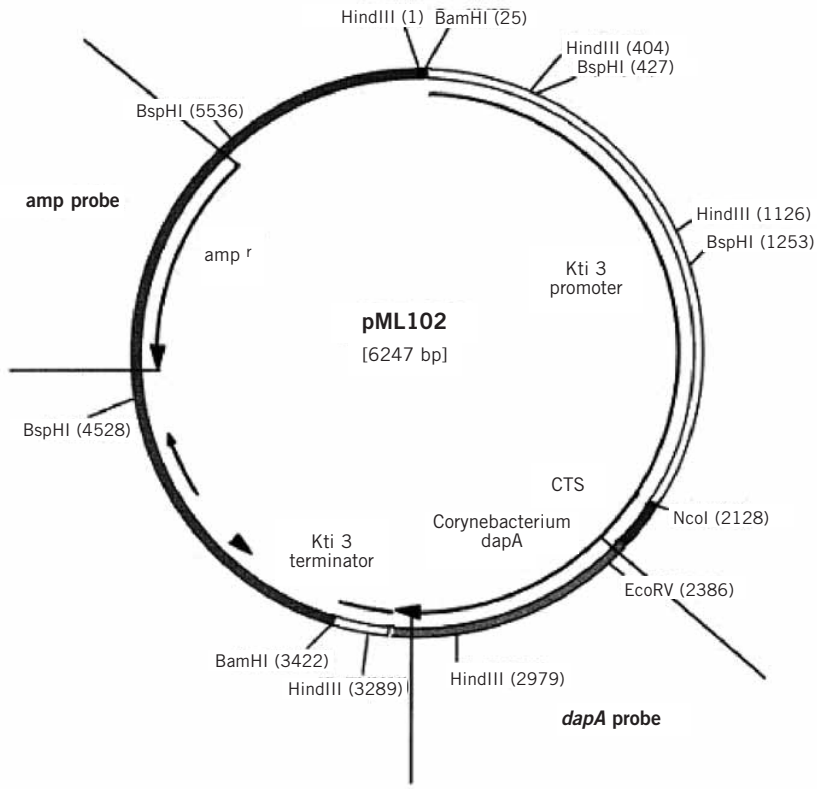


Figure 4: Plasmid map of pML102. Figure indicates the location of hybridisation probes and restriction enzyme sites used for Southern blot analysis of high oleic soybeans.

Interpretation of this DNA hybridisation pattern in Figure 2 suggests that in the original transformation event (event 260-05) the *GmFad 2-1* construct was integrated at two different loci in the soybean genome. Line G168 contains one of the loci (designated *locus A*) consisting of two linked *GmFad 2-1* genes as indicated by the two hybridising fragments of 14.0 kb and 4.5 kb. Line G175 contains the second locus (*locus B*) consisting of a single *GmFad 2-1* gene. G94 contains both loci and thus showed all three hybridising fragments. Only G168 and G94 were selected for further analysis because these showed the desired phenotype of high oleic acid content. Southern blotting of G94 also showed the presence of the *dapA* gene responsible for the increased lysine phenotype.

As G94 plants contained both *locus A* and *locus B*, an additional round of selection was necessary on the segregating R2 plants to isolate plants containing *locus A* and not *locus B*. Southern blot analysis on R2 leaf tissue grown from G94 R2 seed identified two sub-lines, G94-1 and G94-19, that contained *locus A* (Figure 3) without locus B, which had been removed through segregation. *Locus B* was not further characterised.

The two sub-lines, G94-1, G94-19 and line G168, identified as containing the *GmFad 2-1* locus A, were selected as the high oleic acid soybeans for subsequent analyses. The application for food use relates to these sub-lines only. None of these three lines express the high lysine trait.

Molecular characterisation of the DNA insertion in sub-lines G94-1, G94-19 and G168

To fully characterise the insertion in G94-1, G94-19 and G168, six different DNA hybridisation probes based on the genetic fragments in pBS43 (Figure 1) and pML102 (Figure 4) were used for Southern blot analysis. The six probes used were *GmFad 2-1*, phaseolin 3', GUS, 35S

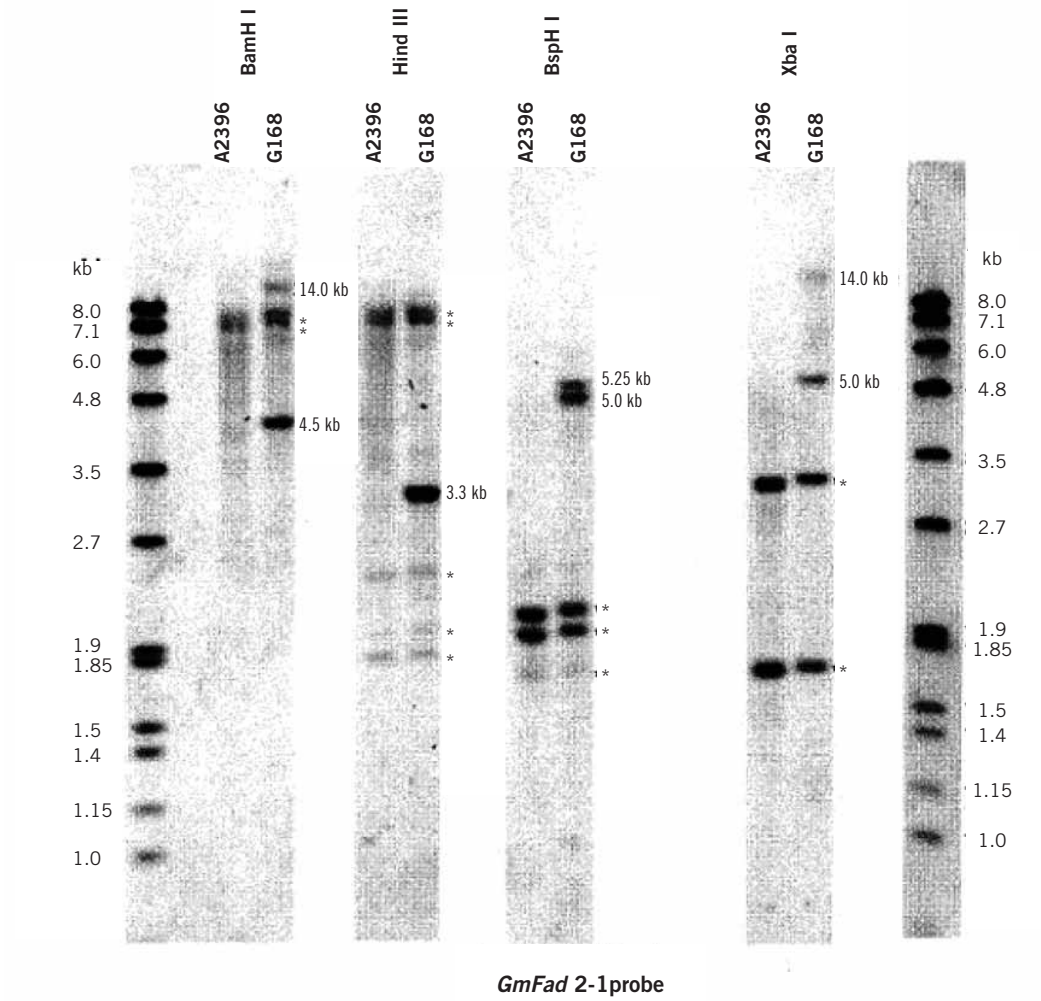


Figure 5a. Southern blot analysis of DNA isolated from R6 leaf tissue of high oleic soybean sub-line G168 and from control line A2396. Genomic DNA was digested with the indicated enzymes and hybridised with the *GmFad 2-1* probe. The underlined molecular weight sizes indicate the sizes of the hybridising transgene for each digest and the asterisks indicated the hybridising endogenous *GmFad 2-1* bands.

promoter, Amp, and *dapA*. Genomic DNA was isolated from R6 leaf tissue from two plants each of G94-1, G94-19, and G168 and the control line A2396. The DNA was digested with six different restriction enzymes to fully characterise the insertions. The results of the Southern blot analysis are presented in Figures 5a and 5b. Table 3 shows the sizes of DNA fragments expected from the different digestions, if it is assumed that one intact copy of plasmid pBS43 was inserted into the genome. For comparison, the sizes of fragments actually obtained in the Southern blot analyses are shown in Table 4.

From the information obtained in these Southern blot analyses, it was possible to deduce a map of the inserted DNA present in the soybean lines (Figures 6a and 6b).

Characterisation of the R6 generation also revealed that a truncated *dapA* gene had been integrated into another locus in the genome of the G94 sub-lines and G168 (*locus C*). These Southern data are not presented in this case study.

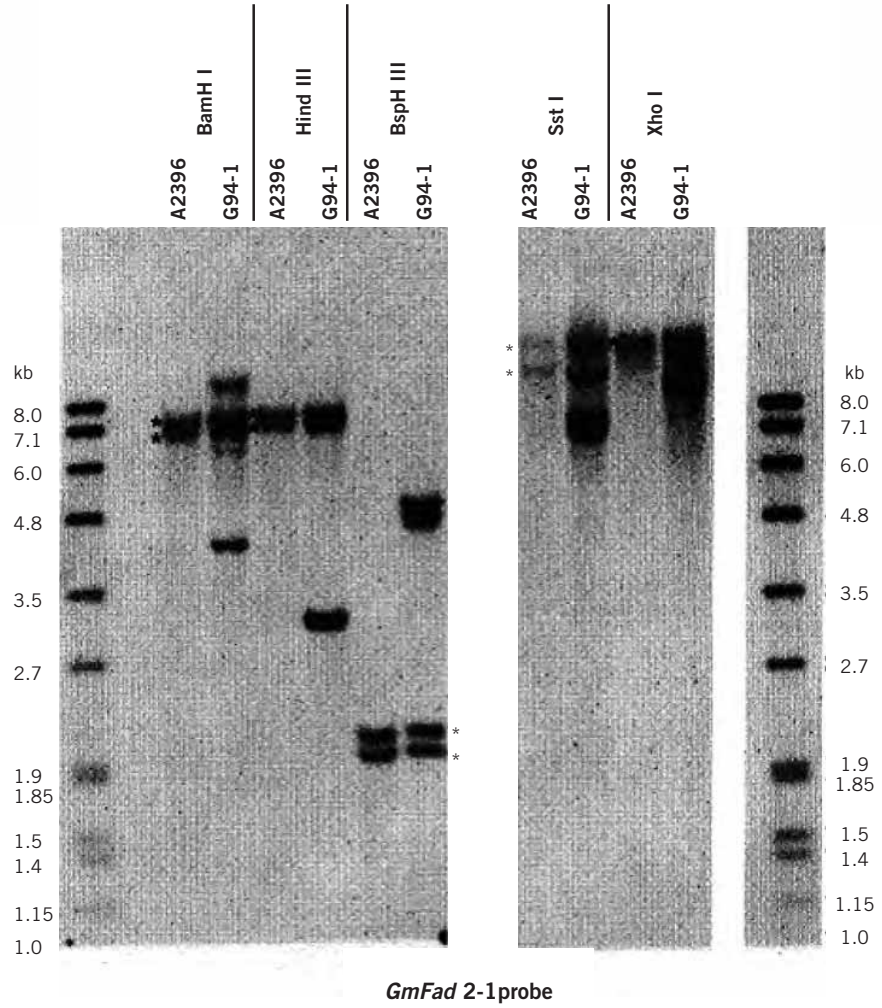


Figure 5b. Southern blot analysis of DNA isolated from R6 leaf tissue of high oleic acid soybean sub-line G94-1 and from control line A2396. Genomic DNA was hybridised with the *GmFad 2-1* probe.

Table 3. Expected fragment sizes (kb). Summary chart of expected hybridising fragment sizes based on the sequence of pBS43 if inserted into the genome as one intact copy

Restriction Enzyme	***Hybridisation Probe***				
	GmFad 2-1	Phaseolin 3'	GUS	35S Promoter	*amp*
HindIII	3.3	3.3	7.0	7.0	7.0
BamHI	Border fragment	Border fragment	Border fragment	Border fragment	Border fragment
BspHI	5.25	5.25	5.25	5.25	1.0
SstI	5.1	2.5	2.7	2.5	5.1
XbaI	9.1	1.2	9.1	9.1	9.1
XhoI	Border fragment	Border fragment	Border fragment	Border fragment	Border fragment

Table 4. Actual fragment sizes (kb)[1]. Summary chart of Southern blot results describing the DNA fragment sizes that hybridised to the indicated probes when high oleic soybean genomic DNA was digested with the listed restriction enzymes

Restriction Enzyme	*Hybridisation Probe*				
	GmFad 2-1	Phaseolin 3'	GUS	35S Promoter	*amp*
HindIII	**3.3**[2]	**3.3**	6.5	6.5	6.5 4.2 3.3
BamHI	14.0 4.5	14.0 4.5	6.5	6.5	14 6.5 2.8
BspHI	5.25 5.0	5.25 5.0	5.25 5.0	5.25 5.0	1.4 **1.0**
SstI		**2.5**	2.7 1.7	**2.5**	
XbaI	14.0 5.0	**1.5**	6.7	6.7	
XhoI			4.4		

[1] *Hybridising fragments larger than 10 kb should be considered as approximate sizes due to the limitations of the gel system for separating large fragments.*

[2] *Fragment sizes that are bold and underlined indicate two copies of the fragment are released by digestion with the listed enzyme. These fragments may give stronger hybridisation signals.*

Figure 6a and 6b: Schematic diagram of insert at locus A in high oleic acid soybeans. The top section of each diagram details the inserted genetic elements from the plasmids and their orientation. The bottom section diagrams the hybridising fragments for each restriction enzyme shown in Table 4. The inserted DNA is drawn to scale whereas the bordering soybean genomic DNA is not drawn to scale.

Summary of 'Locus A'

The mapping of *locus A* shows that one copy of pBS43, opened in the *bla* gene, inserted intact into the genome. A second copy of pBS43, opened in the *uidA* gene, inserted as an inverted repeat relative to the first copy. At the 5' end of *locus A*, proceeding from the soybean genomic DNA junction to the first copy of pBS43, a fragment of pML102, containing only the vector region with the *bla* gene, was inserted. Therefore, the insertion at locus A consists of two intact copies of the *GmFad 2-1* expression cassette, one intact copy of the *uidA* expression cassette and a truncated copy of the *uidA* gene, and at least two intact copies of the *bla* gene plus one truncated copy.

A series of Northern blots (for RNA expression), Western blots (for protein expression) and amino acid profiles were done on sub-lines G94-1, G94-19 and G168 to confirm that the functional *dapA* gene at *locus B* was absent. However, additional Southern blots (data not shown), using a *dapA* probe, indicated that a truncated *dapA* gene expression cassette had become integrated into another locus in the genome (*locus C*). This locus segregates independently of *locus A*. The truncated *dapA* gene is non-functional as indicated by Northern, Western and amino acid analyses.

Stability of the genetic changes

Sub-lines G94-1, G94-19 and G168 differ from the parent line A2396 in that the fatty acid profile has been altered to produce oil containing about 82-85% oleic acid with consequent low levels of linoleic (< 1%) and linolenic acids (< 2.5%). This compares to a range of 19–30% oleic acid reported for standard edible soybean oil (Codex Alimentarius 1989).

To evaluate the genetic and phenotypic stability of the sub lines, genomic DNA from a number of generations of high oleic acid soybeans, homozygous for the *GmFad 2-1 locus A*, were subject to detailed Southern blot analyses. The applicant reports that sub lines G94-1, G94-19 and G168 had been kept separate for six generations and all were shown to maintain identical Southern banding patterns over that period. Analysis of the oleic acid content of seeds from eight different generations also showed that the fatty acid phenotype was stable over this period, with average oleic acid content greater than 80%. In addition, the high oleic acid trait is also reported by the applicant to be stable over a number of different growing environments when compared to the elite parent line and a high oleic acid

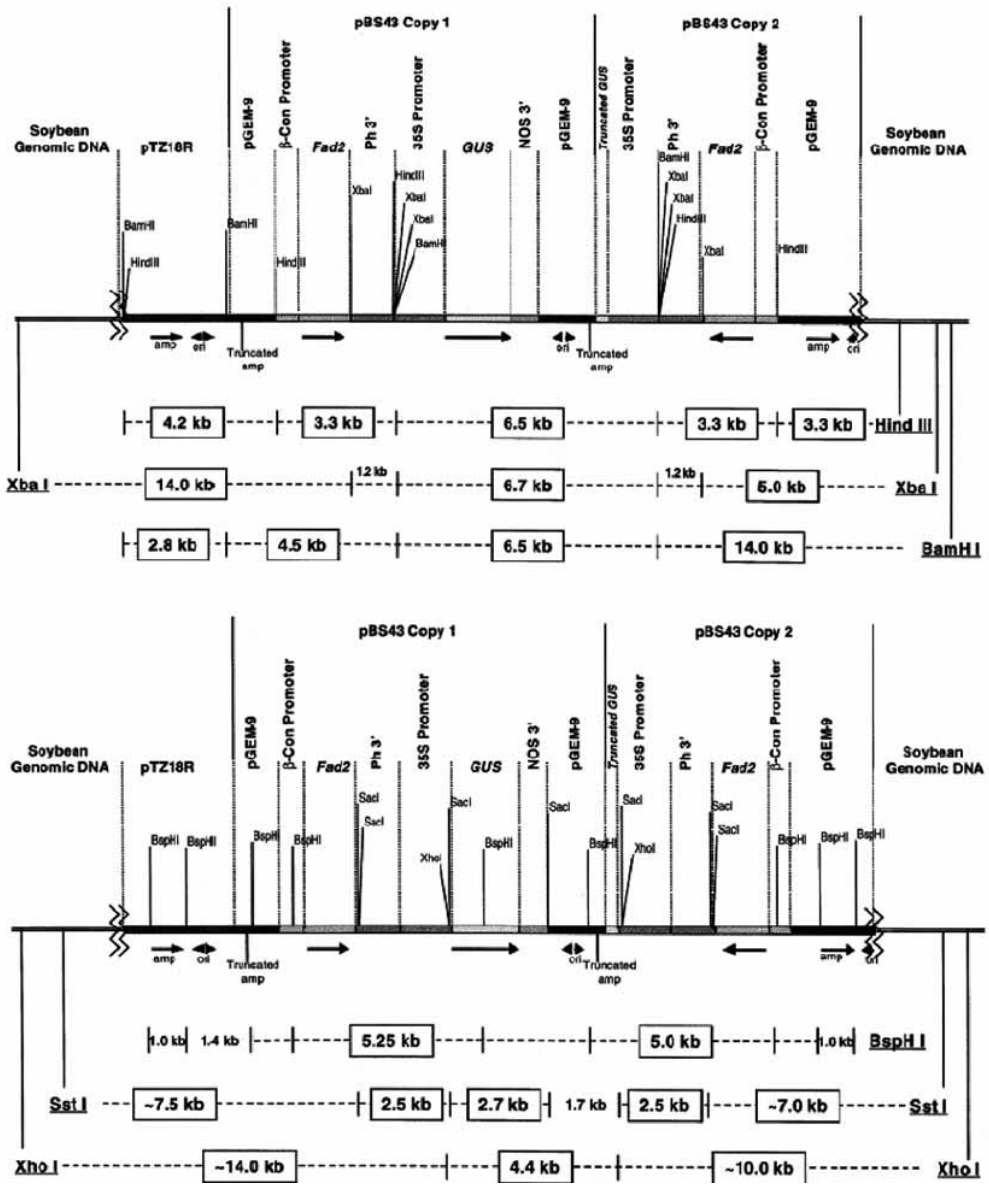


Figure 6a (top) and 6b (bottom). Schematic diagram of insert at *locus A* in high oleic acid soybeans. The top section of each diagram details the inserted genetic elements from the plasmids and their orientation. The bottom section diagrams the hybridising fragments for each restriction enzyme shown in Table 3.4. The inserted DNA is drawn to scale whereas the bordering soybean genomic DNA is not drawn to scale.

soybean line derived through conventional breeding methods.

Conclusion

The *GmFad 2-1* genes in the three sub-lines of high oleic acid soybeans are stably integrated and all three lines are phenotypically and genetically stable over multiple generations and in various environments.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA per se (WHO 1993). There have been concerns expressed, however, that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to

microorganisms present in the human digestive tract and that this could compromise the therapeutic use of antibiotics.

This section of the case study therefore concentrates on evaluating the human health impact of the potential transfer of antibiotic resistance genes from high oleic acid soybeans to microorganisms present in the human digestive tract.

The two plasmids used to transform soybean line A2396 – pBS43 and pML102 – both contained a copy of the *bla* gene under the control of a bacterial promoter. The *bla* gene encodes the enzyme β-lactamase and confers resistance to a number of β-lactam antibiotics such as penicillin and ampicillin. Molecular characterisation of the high oleic acid soybean lines has confirmed the presence of two intact copies of the bla gene along with its bacterial promoter. The *bla* gene is not itself expressed in the high oleic acid soybean lines (see Section 6.7).

The first issue that must be considered in relation to the presence of an intact bla gene in the high oleic acid soybeans is the probability that this gene would be successfully transferred to, and expressed in, microorganisms present in the human digestive tract. The following steps would be necessary for this to occur:

- Excision of DNA fragments containing the bla gene and its bacterial promoter;
- Survival of DNA fragments containing the bla gene in the digestive tract;
- Natural transformation of bacteria inhabiting the digestive tract;
- Survival of the bacterial restriction system by the DNA fragment containing the bla gene;
- Stable integration of the DNA fragment containing the bla gene into the bacterial chromosome or plasmid;
- Maintenance and expression of bla gene by the bacteria.

The transfer of a functional bla gene to microorganisms in the human digestive tract is considered to be highly unlikely because of the number and complexity of the steps that would need to take place consecutively.

The second and most important issue that must be considered is the potential impact on human health in the unlikely event successful transfer of a functional bla gene to microorganisms in the human digestive tract did occur.

In the case of the bla gene, the human health impacts are considered to be negligible because ampicillin-resistant bacteria are commonly found in the digestive tract of healthy individuals (Calva *et al*., 1996) as well as diseased patients (Neu 1992). Therefore, the additive effect of a bla gene from the high oleic acid soybeans being taken up and expressed by microorganisms of the human digestive tract would be insignificant compared to the population of ampicillin resistant bacteria already naturally present. In addition, ampicillin has now largely been replaced by more potent forms of β-lactam antibiotics or is only used in combination with drugs that work to inactivate β-lactamase (Walsh 2000).

Conclusion

It is extremely unlikely that the ampicillin resistance gene will transfer from high oleic acid soybeans to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. In the highly unlikely event that the ampicillin resistance gene was transferred to bacteria in the human digestive tract the human health impacts would be negligible because ampicillin resistant bacteria are already commonly found in the human gut and in the environment and ampicillin is rarely used clinically.

Characterization of novel protein

Biochemical function and phenotypic effects

δ-12 desaturase

The synthesis of polyunsaturated fatty acids in developing oilseeds is catalysed by two membrane-associated desaturases that sequentially add a second and third double bond to oleic acid (Kinney, 1994). The pathway for the synthesis of long chain fatty acids in plants is depicted in the introductory chapter.

The second double bond, converting oleic acid to linoleic acid, is added at the δ-12 (n-6) position by a δ-12 desaturase, encoded by the *GmFad 2-1* gene (Okuley *et al.*, 1994, Heppard *et al.*, 1996). The third double bond, converting linoleic acid to linolenic acid, is added at the n-3 (δ-15) position by an n-3 desaturase, encoded by the *GmFad 3* gene (Yadav *et al.*, 1993). The *GmFad 2-1* gene used to genetically modify the soybeans is itself derived from soybean.

Dihydrodipicolinic acid synthase

Dihydrodipicolinic acid synthase (DHDPS) is responsible for catalysing the first step in the metabolic pathway for the synthesis of the essential amino acid lysine (Brock *et al.*, 1984). DHDPS catalyses the condensation of

aspartate semi-aldehyde with pyruvate to form 2,3-dihydrodipicolinate. The reaction takes place in the chloroplast of higher plants as well as in many bacteria. In plants, DHDPS is inhibited by lysine and is the major regulatory enzyme of lysine biosynthesis. Animals are incapable of synthesising lysine; therefore they must obtain their lysine through dietary sources.

β-glucuronidase

The *uidA* gene from *E. coli* encodes the enzyme β-glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31), which is an acid hydrolase that catalyses the cleavage of a wide variety of β-glucuronides. Many glucuronide substrates can be used for spectrophotometric, fluorometric and histochemical analyses. Very little, if any, β-glucuronidase activity has been detected in higher plants (Jefferson *et al.*, 1986), therefore fusions of the *uidA* gene to plant genes or promoters can be used as a visual marker of plant transformation. In the case of plants that have been transformed with the *uidA* gene, the colourimetric substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide is used as an indicator of β-glucuronidase activity.

β-lactamase

The bacterial *bla* gene codes for the enzyme β-lactamase and confers resistance to some β-lactam antibiotics, such as penicillin and ampicillin. The gene is used as a marker to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells. Only those bacterial cells that express the β-lactamase will grow in the presence of antibiotic. As the *bla* gene is under the control of a bacterial promoter it would not be expected to be expressed in transformed plant cells.

Protein expression analyses

δ-12 desaturase

Northern blot analysis, using the *GmFad 2-1* gene as a probe, was done on RNA isolated from developing R4 seeds of the high oleic acid soybeans at the time when the endogenous *GmFad 2-1* would normally be expressed (Figure 7). The δ-conglycinin promoter, linked to the transferred copy of the *GmFad 2-1* gene, is also active during this period. The data shows that seeds containing *GmFad 2-1 locus A* (G94-1, G168) do not have any detectable *GmFad 2-1* mRNA, whereas, seeds that contain the *GmFad 2-1 locus B* (G175) or seeds that only contain the endogenous *GmFad 2-1* gene (G90) have significant levels of mRNA. This demonstrates that neither of the *GmFad 2-1* genes is transcribed in the high oleic acid soybeans.

Dihydrodipicolinic acid synthase

Northern blot analysis, using the dapA probe, was done on RNA isolated from R6 leaves and R4 immature seeds of the high oleic acid soybeans (Figure 8). The data show that there is no detectable expression of dapA

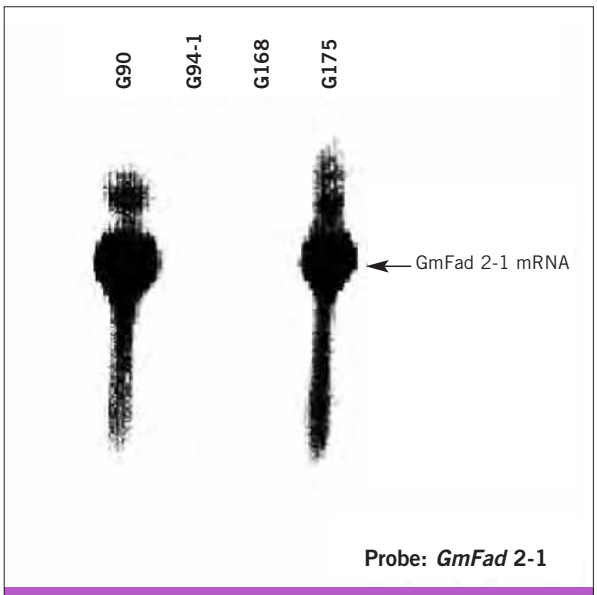


Figure 7. GmFad 2-1 Northern blot analysis on RNA isolated from developing R4 seeds at 20 days after flowering. G90 contains only the endogenous GmFad 2-1 gene and was used as a wild-type control. G94-1 and G168 contain the *GmFad 2-1 locus A* and G175 contains the *GmFad 2-1 locus B.*

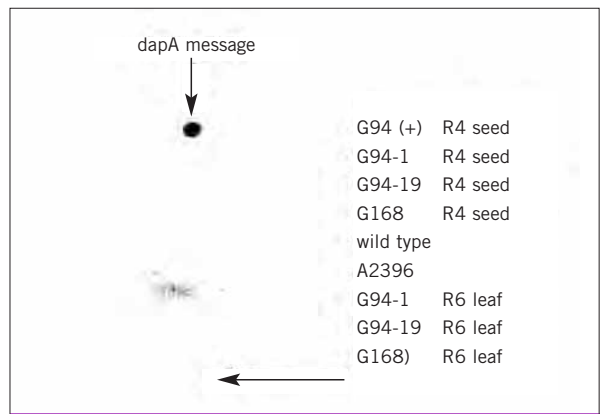


Figure 8. Northern blot analysis of high oleic soybeans. The blot was probed with the dapA coding region. Seed G94 contained the dapA gene and was used as a positive control. Two negative controls were used and labelled as wild type and A2396. The top of the gel is to the right and the bottom is to the left.

mRNA in sub-lines G94-1, G94-19 and G168. Western blot analysis, using a polyclonal anti-Corynebacterium DHDPS antibody, was done on total protein isolated from leaves and seeds of the three sub-lines. The data show that DHDPS protein can only be detected in seeds of the high lysine positive control line and not in any of the high oleic acid sub-lines under consideration.

Amino acid analyses were done on three replicates of each of the high oleic acid soybean sub-lines. These show that there are no differences in the lysine levels of the high oleic acid soybeans when compared to the parental soybean line (A2396).

β-glucuronidase

An intact *uidA* expression cassette is present in sub lines G94-1, G94-19 and G168, however, colourimetric analyses of R6 seeds and leaves from these lines show that the *uidA* gene is not expressed (Figure 9). The original transformant, line 260-05, was selected on the basis of its GUS expression therefore the *uidA* gene has become 'switched off' in subsequent generations. The applicant has not speculated as to the reason for the inactivation of the *uidA* gene, however, the inactivation of transgenes is relatively common in plants (Kilby *et al.*, 1992, Ingelbrecht *et al.*, 1994, Brusslan and Tobin, 1995).

β-lactamase

All of the lines derived from event 260-05, which contain only *GmFad 2-1 locus A*, also contain two intact copies of the *bla* gene. These two copies are under the control of a bacterial promoter and, therefore, should not be expressed in the plant cell. To confirm this, the activity of β-lactamase was measured in cell free extracts of leaf tissue from sub-line G94-1. The results of this study, which show that there is no detectable β-lactamase activity in sub-line G94-1, confirm that the *bla* gene is not expressed in plant cells (Figure 10).

Assessment of possible toxicity

If the GM food differs from its traditional counterpart by the presence of one or a few novel proteins, it is usually possible to assess the potential toxicity of these proteins in a manner analogous to traditional toxicity testing (WHO 2000). That is, the assessment is applied to the novel protein itself, rather than the whole food.

In considering the potential toxicity of a novel protein it is first important to determine whether it is likely to be present in the food as consumed, and thus whether exposure is likely[37]. Once likely human exposure to a novel protein is established, a number of different pieces of information can collectively be used to demonstrate there is a reasonable certainty that no harm will result from that exposure.

An assessment of potential toxicity of a novel protein should consider the following:

- Whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food;

[37] Even if it can be demonstrated that a protein will not be present in the edible portion, proteins known to be toxic to humans should never be deliberately introduced into another organism to be used for food because of the risk of accidental carryover into the edible portion.

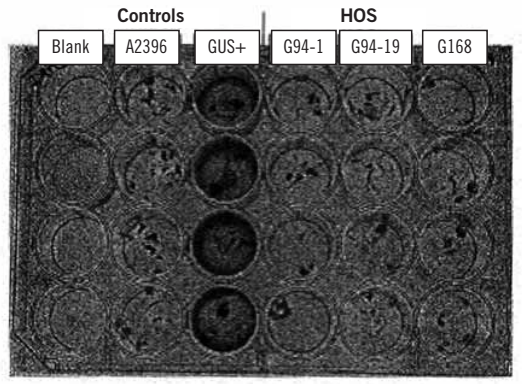
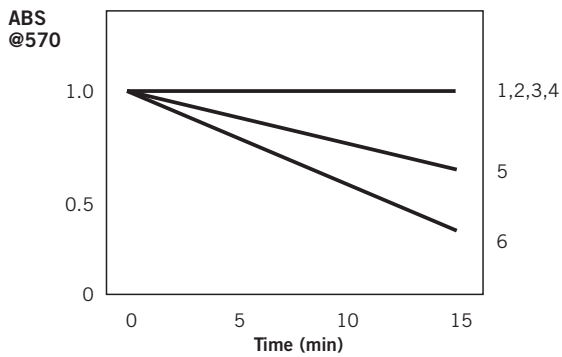


Figure 9. Colorimeteric GUS enzyme assay analysis on R6 seeds of high oleic acid soybean sub-lines G94-1, G94-19 and G168 and positive and negative (A2396) control lines. The positive control is a well-characterised GUS positive soybean line from a different transformation event. The dark colour of the solution in the wells indicates GUS enzyme activity.



1 = 50 µg BSA; 2 = 500µg A2396; 3 = 500µg G94-1;
4 = 2500 µg G94-1; 5 = 50 µg *E. coli*; 6 = 100 µg *E. coli*

Figure 10. b-lactamase activity in high oleic soybeans, elite control A2396 soybeans and in *E. coli* transformed with pBS43.

- Whether there is any amino acid sequence similarity between the novel protein and known protein toxins and anti-nutrients;
- Whether the novel protein causes any adverse effects in acute oral toxicity testing;
- Whether the novel protein is resistant to heat and/or processing;
- Whether the novel protein is resistant to degradation in simulated digestion models.

It should be noted that, unlike many other substances that are added to foods, the majority of proteins have a predictable metabolic fate in the digestive system, that is, they are typically broken down into their constituent amino acids and then assimilated. For novel proteins, it is therefore important to establish that they will behave like any other dietary protein. One method that can be used to demonstrate this is an in vitro digestibility assay. This assay should be able to establish if a novel protein has any characteristics unusual in dietary protein, such as resistance to digestive fluids.

Acute oral toxicity testing is an important component of the safety assessment of novel proteins and is particularly useful in circumstances where there is no prior history of safe consumption of the protein. Acute tests should be sufficient since - if toxic - proteins are known to act via acute mechanisms and laboratory animals have been shown to exhibit acute toxic effects from exposure to proteins known to be toxic to humans (Sjoblad *et al.*, 1992). The acute toxicity tests are done using purified protein that is administered at very high dose levels, usually orders of magnitude above what the human exposure level would be. Ideally, the protein to be tested should be that which has been directly purified from the new organism. Where this is not possible, usually because it is difficult to obtain sufficient quantities of purified protein, it is essential to ensure that the protein tested is biochemically and functionally equivalent to that present in the GM food.

If a novel protein is found to have no significant sequence similarities to known protein toxins, is not stable to heat and/or processing and is readily digested in conditions that mimic mammalian digestion and either has a prior history of safe human consumption and/or does not cause any toxic effects in acute toxicity testing then it can be reasonably concluded that the protein is non-toxic to humans and no further toxicological investigations would be required.

If a novel protein fails one or more of the criteria discussed above then further investigation of the novel protein may be required. For example, if adverse effects were noted in acute toxicity testing then additional toxicity testing would be required to determine a safe level of human exposure.

As part of the assessment of the potential toxicity of a novel protein it is important to also determine if the activity of the novel protein in the organism is likely to produce any secondary effects, such as the accumulation of other substances. If other substances are found to accumulate as a result of the activity of a novel protein, *e.g.*, the accumulation of a metabolite as a result of the detoxification of a herbicide in a plant, it is important to also include an assessment of the potential toxicity of such substances.

Assessment of possible allergenicity

Virtually all food allergens are proteins, but only a small fraction of the many proteins found in food are allergenic. Therefore, even though foods can contain tens of thousands of different proteins, relatively few are allergenic. As the use of recombinant-DNA techniques can result in additional protein diversity being added to the food supply, the potential allergenicity of any new protein should be a part of the safety assessment. It should be noted however that additional protein diversity could also be introduced into the food supply through conventional breeding techniques.

The prediction of the allergenic potential of a novel protein is not a simple matter and there are presently no validated animal models for the assessment of allergenicity. Because of this, the potential for a novel protein to be allergenic must be evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity.

The assessment focuses on the source of the novel protein, any significant amino acid similarity between the novel protein and that of known allergens, and the structural properties of the novel protein, including susceptibility to digestion. Applying such criteria systematically provides reasonable evidence about the potential of a novel protein to act as an allergen (Lehrer and Reese 1998; Jones and Maryanski 1991).

The source of the novel protein and its amino acid sequence similarity to known allergens are key considerations in the allergenicity assessment. If the novel protein comes from a source known to be allergenic or has sequence similarity to a known allergen, further immunological testing, using sera from

individuals with a clinically validated allergy to the source of the protein, can be used to determine if the novel protein is likely to illicit an allergic response in affected individuals. A negative result may necessitate additional testing, such as skin tests in appropriate subjects.

Resistance to digestion has been observed in several food allergens, therefore such information will also be useful in making an overall determination about the potential for a novel protein to be allergenic to humans. The ability of food allergens to reach and cross the intestinal mucosal barrier in immunologically intact form appears to be a prerequisite to allergenicity (Metcalfe *et al.*, 1996). Simulated gastric and intestinal digestive models of mammalian digestion are typically used to assess the digestive stability of proteins (Astwood *et al.*, 1996).

As with potential toxicity, exposure to the novel protein is also an important consideration, which will contribute to an overall conclusion about the potential for a novel protein to be allergenic to humans. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of food processing which would be applied and its effects on the presence of the protein in the final food product. A classic example where this is relevant is in the case of refined oils, which typically do not contain any detectable protein.

Compositional analyses of key components, evaluation of metabolites, food processing and nutritional modification

A comparative approach, focussing on the determination of similarities and differences between the GM food and its conventional counterpart, aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO 2000). The compositional analysis, where the key nutrients, key toxicants and anti-nutrients are measured in the GM food, is an important part of the comparative assessment. The key nutrients and toxicants/anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. These may be major constituents (*e.g.*, fats, proteins, carbohydrates) or minor components (*e.g.*, minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (*e.g.*, solanine in potatoes if the level is increased). The key components of soybeans that should be considered in the comparison include protein, fat, carbohydrates, amino acids, fatty acids, phytic acid, trypsin inhibitors, lectins and isoflavones (OECD 2001). The composition of the high oleic acid soybeans was compared to that of the elite soybean line from which they were derived (A2396).

Field studies and data collection

Two separate field studies of the high oleic acid soybeans were conducted. In the first study, lines G94-1 and G94-19 were grown at two locations in the United States: Slater, Iowa, and Isabella, Puerto Rico during the summer of 1995 and the Winter of 1995/1996. Seeds, representing the R4 and R5 generation, were analysed from each location. Values were obtained from duplicate assays on single samples from each of the four locations. Analyses were done of raffinose, stacchyose and phytic acid content as well as isoflavone content. In the second study conducted in the summer of 1996, lines G94-1, G94-19 and G168 were grown in parallel with the parental line A2396 at four locations in the United States: Redwood Falls, Minnesota, Kalamazoo, Michigan, Prairie City, Iowa and Cedar Rapids, Iowa. Seeds, representing the R6 generation, were analysed from each of the four locations. Values were obtained from duplicate assays on three replicates from each of the four locations. Analyses were done of proximate, trypsin inhibitor, amino acid, fatty acid, vitamin and mineral, and tocopherol content.

Key nutrients

Proximate analyses

Proximate analysis includes the measurement of crude fat/oil, protein, fibre, and ash content and is done to determine if there have been any changes to the major constituents of the soybean seed. The results of the proximate analysis are presented in Table 5.

The results show that there are no significant differences in proximate composition between the parental soybean line and the high oleic acid soybeans. The values obtained are also comparable to those reported in the literature for soybeans.

Amino acid composition

Amino acid content was determined for 17 out of the 20 amino acids. The three amino acids not analysed were

Table 5. Proximate content[1] of control and high oleic acid soybeans

	Parental control	*High oleic acid lines*	*Literature range*
	(g/100 g dry weight unless noted)		
Moisture (g/100 g fresh wt)	7.69 (7.00-8.20)	7.85 (7.20-8.40)	7-11
Crude fat/oil	25.37 (21.62-28.29)	23.90 (19.74-29.28)	13.2-22.5
Protein	40.11 (38.41-41.68)	40.76 (38.85-42.97)	36.9-46.4
Fibre	6.11 (5.44-7.14)	6.76 (5.00-7.26)	4.7-6.8
Ash	5.13 (4.53-5.85)	4.81 (4.13-5.54)	4.61-5.37

[1] *Mean values, the range in brackets.*

Table 6. Amino acid content[1] of parental and high oleic acid soybeans

Amino acid	*Parental control*	*High oleic acid lines*	*Literature range*
	(g/100 g dry weight)		
Tryptophan	0.44 (0.41-0.46)	0.47 (0.42-0.51)	0.53-0.54
Lysine	2.45 (2.27-2.63)	2.38 (2.17-2.67)	2.35-2.86
Histidine	0.96 (0.90-1.05)	0.93 (0.83-1.09)	0.89-1.08
Arginine	2.64 (2.42-2.91)	2.64 (2.37-2.88)	2.45-3.49
Aspartic acid	4.3 (3.98-4.58)	4.45 (4.14-4.93)	3.87-4.98
Threonine	1.37 (1.24-1.50)	1.52 (1.38-1.70)	1.33-1.79
Serine	1.79 (1.61-1.95)	1.84 (1.65-2.02)	1.81-2.32
Glutamic acid	7.13 (6.58-7.81)	7.03 (6.50-7.79)	6.10-8.72
Cysteine	0.55 (0.51-0.60)	0.58 (0.52-0.71)	0.56-0.66
Glycine	1.57 (1.44-1.68)	1.71 (1.56-1.85)	1.88-2.02
Alanine	1.54 (1.43-1.68)	1.67 (1.50-1.84)	1.49-1.87
Valine	1.73 (1.61-1.86)	1.84 (1.58-2.05)	1.52-2.24
Methionine	0.47 (0.44-0.50)	0.54 (0.47-0.60)	0.49-0.66
Isoleucine	1.72 (1.48-1.87)	1.76 (1.54-2.00)	1.46-2.12
Leucine	2.86 (2.64-3.05)	2.91 (2.70-3.18)	2.71-3.20
Tyrosine	1.45 (1.35-1.54)	1.51 (1.38-1.62)	1.12-1.62
Phenylalanine	1.82 (1.71-1.97)	1.86 (1.72-2.03)	1.70-2.08

[1] *Mean values, the range in brackets.*

proline, asparagine and glutamine. A summary of the results of the amino acid analysis appears in Table 6.

No significant differences were observed in amino acid content between the parental line and the high oleic acid soybeans for any of the 17 amino acids analysed. The values determined were comparable to the literature reported ranges.

Fatty acid composition

A complete fatty acid analysis of oil from the high oleic acid soybean lines G94-1 and G94-19 and control soybean lines grown in field trials in 1995/1996 was done and compared to the ranges specified by Codex Alimentarius for soybean oil. The results of the analysis are presented in Table 7.

A further, but more limited analysis of fatty acid content was done on all three high oleic acid soybean lines and the parental control soybean line grown in field trials in 1996. The results of the analysis are presented in Table 8.

The results from the two separate analyses demonstrate that the high oleic acid soybeans differ significantly from the parental soybean line in the levels of oleic, linoleic, linolenic and palmitic acid present in the oil. Oleic acid levels have been significantly increased and this has resulted in concomitant decreases in the levels of palmitic, linoleic and linolenic acids. The levels of other fatty acids present in the oil were similar between the parental and high oleic acid soybean lines and were comparable to the Codex

Table 7. Complete fatty acid analysis of control and high oleic acid soybean lines from 1995/96 field trials

Fatty acid	*Parental control*	*G94-1*	*G94-19*	*Codex range*
	(g/100 g fatty acid, mean values presented, ranges not provided)			
C14:0 myristic	<0.1	<0.1	<0.1	<0.5
C16:0 palmitic	10.1	6.3[1]	6.6	7.0-14.0
C16:1 palmitoleic	0.1	0.12	0.12	<0.5
C16:2 hexadienoic	<0.1	<0.1	<0.1	
C16:3 hexatrienoic	<0.1	<0.1	<0.1	
C18:0 stearic	3.2	3.7	3.6	1.4-5.5
C18:1 oleic	14.7	84.6	84.9	19.0-30.0
C18:2 (9,12) linoleic	61.6	0.9	0.6	44.0-62.0
C18:2 (9, 15) linoleic	<0.1	0.8	0.7	
C18:3 linolenic	9.5	2.4	1.9	4.0-11.0
C20:0 arachidic	0.2	0.4	0.5	<0.1
C20:1 eicosenoic	0.2	0.4	0.4	<0.1
C20:2 eicosadienoic	not done	not done	not done	
C22:0 behenic	0.3	0.4	0.5	<0.5
C22:1 erucic	<0.1	<0.1	<0.1	
C24:0 lignoceric	0.1	0.1	0.2	

[1] *Complete fatty acid analysis of control and high oleic acid soybean lines from 1995/96 field trials.*

Table 8. Fatty acid composition1 of oil from high oleic acid and control soybean lines from 1996 field trials

Fatty acid	*Parental control*	*High oleic acid lines*	*Literature range*
	(g/100 g fatty acid)		
C16:0 palmitic	10.25 (9.94-10.59)	6.55 (6.22-6.96)	7-12
C18:0 stearic	3.95 (3.57-4.27)	3.43 (3.04-3.81)	2-5.5
C18:1 oleic	23.09 (22.07-23.91)	83.84 (80.02-85.38)	20-50
C18:2 linoleic	55.36 (53.61-56.48)	2.23 (1.19-4.83)	35-60
C18:2 9,15 linoleic isomer	0.00	0.48 (0.37-0.56)	-
C18:3 linolenic	7.35 (6.81-8.35)	3.47 (2.87-4.51)	2-13

[1] *Mean values, the range in brackets.*

Alimentarius ranges for soybean oil. High levels of oleic acid are commonly consumed in other premium edible oils (*e.g.*, olive oil, high oleic acid sunflower and canola oils). The increased oleic acid levels do not pose a safety concern.

In addition to the expected changes to the fatty acid composition of oil from the high oleic acid soybean lines, a trace amount (less than 1% of the total fatty acid content) of the 9,15 isomer of linoleic acid (cis-9, cis-15-octadecadeinoic acid), normally found only in hydrogenated soybean oils and butterfat, was also detected. This isomer is not present in the oil of the parental soybean line A2396.

The applicant speculates that the presence of the isomer is the result of activity of a δ-15 (n-3) desaturase (GmFad3), which normally inserts a δ-15 double bond into 9,12-linoleic acid. In the transgenic plants, the linoleic acid content is reduced from >50% of the total fatty acids to <2% and therefore they speculate that the GmFad3 enzyme probably creates a small amount of the isomer by putting a δ-15 double bond into 9-oleic acid. The applicant provided data to support this hypothesis where the high oleic acid soybeans were crossed with a soybean containing a suppressed *GmFad3* gene. In the resulting progeny, the isomer is either reduced or virtually eliminated.

The applicant provided data on the occurrence of the 9,15 isomer of linoleic acid in commonly used oils and fats for frying and baking in Europe. This data is presented in Table 9.

Table 9. Occurrence of the 9,15 linoleic acid isomer in commonly used oils and fats for frying and baking

Oil/fat	*Fatty acid composition* (g/ 100 g fatty acid)					
	C16:0	*C18:0*	*C18:1*	*C18:2*	*C18:2 (9,15)*	*C18:3*
Palm olein, partially hydrogenated	20.8	4.0	48.3	22.4	1.3	0.8
Soybean oil, partially hydrogenated	10.8	5.8	44.8	21.4	3.4	0.7
Rapeseed oil, partially hydrogenated	5.6	3.8	72.0	8.9	2.7	1.3
Butter fat	34.8	11.7	26.6	2.6	0.4	0.8

Table 10. Vitamin and mineral content[1] of the control and high oleic acid soybeans

Vitamin or mineral[2]	*Parental control*	*High oleic acid lines*	*Literature range*
	(mg/100 g dry weight unless noted)		
Minerals			
Calcium	264 (245-302)	232 (212-251)	132.7-326.3
Copper	0.64 (0.30-1.00)	0.67 (0.24-1.02)	0.9-5.1
Iron	5.6 (4.2-7.4)	5.8 (3.8-7.9)	3.2-7.9
Magnesium	247 (232-260)	236 (215-261)	
Manganese	2.9 (1.9-4.0)	2.7 (2.2-3.6)	0.4-6.8
Phosphorous	621 (516-742)	636 (501-771)	378-1836
Potassium	1755 (1468-1950)	1689 (1492-1896)	859-1784
Sodium	3.1 (1.1-6.5)	4.3 (2.2-8.7)	
Zinc	4.0 (3.2-4.7)	4.3 (3.0-5.8)	
Vitamins			
Vitamin B6	0.115 (0.098-0.131)	0.125 (0.110-0.141)	
ß-carotene (IU/100 g dry wt)	8 (5-12)	10 (5-16)	
Vitamin B1	0.96 (0.74-1.17)	0.89 (0.63-1.24)	
Vitamin B2	0.29 (0.26-0.30)	0.30 (0.27-0.35)	
Vitamin E (IU/100 g dry wt)	1.2 (1.1-1.6)	1.1 (0.9-1.7)	
Niacin	2.6 (2.28-2.88)	2.74 (2.38-3.15)	
Pantothenic acid	1.051 (0.936-1.132)	0.961 (0.794-1.063)	
Folic acid (°g/100 g dry wt)	274 (184-379)	284 (186-384)	
Tocopherols			
Total	20.11 (18.01-22.50)	18.57 (16.36-21.16)	
Alpha	1.37 (1.11-1.62)	1.32 (1.06-1.62)	1.09-2.84
Beta	0.17 (0.07-0.20)	0.22 (0.15-0.30)	<0.5
Gamma	16.17 (14.03-18.81)	15.42 (13.12-17.58)	15.0-19.1
Delta	1.72 (1.52-2.11)	1.88 (1.61-2.28)	2.46-7.25

[1] *Mean values, the range in brackets.*

[2] *All samples contained less than 0.1 µg/100 g vitamin B12, less than 1.0 mg/100 g vitamin C and less than 5 IU/100 g retinol.*

This data shows that the 9,15 isomer of linoleic acid is commonly found in other edible sources of fat such as butterfat and partially hydrogenated vegetable oils at a range of 0.4-3.4% of the total fatty acids. Therefore, its occurrence in high oleic acid soybean oil at a level of 0.5% of the total fatty acids (representing about 25% of the linoleic acid fraction) is not considered to pose any safety concerns.

Vitamins and minerals

The high oleic acid soybean lines G94-1, G94-19 and G168 and the parental soybean line A2396 were analysed for their mineral and vitamin content including tocopherols. The tocopherols, also known as vitamin E, exist as four isomers (α-, β-, γ-, and δ-tocopherol). The four isomers are not equivalent, with α-tocopherol being the most important in terms of bioactivity. The

Table 11. Isoflavone content[1] of parental and high oleic acid acid soybean lines

Isoflavone	*Parental control*	*High oleic acid lines*	*Literature range*
	(µg/g dry weight)		
Total daidzein	693 (623-762)	612 (525-694)	295-1527
Total genistein	714 (574-854)	724 (548-910)	416-2676
Total glycitein	192 (188-196)	273 (261-287)	149-341

[1] *Mean values, range in brackets.*

Table 12. Lectin content[1] of parental and high oleic acid soybean lines

Lectin	*Parental control*	*High oleic acid lines*	*Literature range*
HU[1]/mg extracted protein	6.36 (4.09-7.90)	7.83 (5.37-9.70)	2.7-12.5
HU/mg total protein	2.98 (2.30-3.90)	3.67 (2.77-4.73)	1.2-6.0
HU/mg sample (FW basis)	1.03 (0.70-1.30)	1.32 (0.97-1.67)	0.5-2.4

[1] *HU = haemagglutinating unit, # mean values, the range in brackets.*

Recommended Daily Intake (RDI) for vitamin E is normally presented as α-tocopherol equivalents. The results of the vitamin and mineral analyses are summarised in Table 10.

No significant differences in mineral or vitamin content, including tocopherols, were observed between the high oleic acid soybeans and the parental soybean line. The mineral content of the high oleic acid soybeans was within the literature reported ranges. With the exception of the tocopherols, literature ranges for vitamin content was not provided. The delta tocopherol content was lower than the literature reported range for both the parental control and high oleic acid soybean lines. The content of the other tocopherols in the high oleic acid soybeans were within the literature reported ranges for soybeans.

Isoflavones

Soybeans naturally contain a number of isoflavone compounds reported to possess biochemical activity, including estrogenic and hypocholesterolemic effects, in mammalian species. Isoflavones (known to include phytoestrogens) have, in the past, also been regarded as anti-nutrients, however, this is no longer universally accepted as isoflavones have also been reported to have beneficial anti-carcinogenic effects. The major isoflavones in soybeans and soybean products include daidzin, genistin, and their corresponding aglycons, daidzein and genistein. Glycitin and glycitein also occur in trace amounts.

High oleic acid soybean lines G94-1 and G94-19 and parental soybean line A2396 were analysed for isoflavone content. The results are summarised in Table 11.

There are no significant differences between the parental soybean and the high oleic acid soybean lines G94-1 and G94-19 in either total daidzein or genistein content which is also within the literature reported ranges for soybeans. In relation to total glycitein content, however, the high oleic acid soybean lines exhibit slightly elevated levels compared to the control. The level reported for total glycitein however is within the literature reported range therefore this slightly elevated level compared to the control is not considered to pose any safety concerns.

Key toxicants

The only naturally occurring toxicants in soybeans are lectins. Lectins are proteins that bind to carbohydrate-containing molecules and which inhibit growth and sometimes cause death in animals. It is reasonable to assume that similar effects would occur in humans. Lectins, however, are rapidly degraded upon heating, and therefore only become an issue when raw soybeans are consumed. There are no human food uses for raw soybeans.

Notwithstanding that there are no human food uses for raw soybeans, the applicant undertook compositional analyses for lectin content of seeds from the high oleic acid soybean lines. The seeds represent the R6 generation of the high oleic acid soybean lines. Lines G94-1, G94-19 and G168 were grown in parallel with the parental line A2396 at four locations in the United States in the summer of 1996. To obtain the data,

Table 13. Anti-nutrient content[1] for parental and high oleic acid soybeans

Anti-nutrient	*Parental control*	*High oleic acid lines*	*Literature range*
Trypsin inhibitor (TIU/mg dry wt)	31.67 (22.84-40.47)	30.20 (14.21-42.43)	26.4-93.2
Phytic acid (g/100 g dry wt)	1.42 (1.32-1.53)	1.42 (1.25-1.69)	1.3-4.1

[1] *Mean values, the range in brackets.*

Table 14. Stacchyose and raffinose content[1] of parental and high oleic acid soybeans

Constituent	*Parental control*	*High oleic acid lines*	*Literature range*
	(µmoles/g dry weight)		
Stacchyose	63 (60-67)	68 (65-75)	44.8-68.8
Raffinose	14 (14-14)	15 (14-16)	8.6-18.5

[1] *Mean values, the range in brackets.*

three replicates were analysed in duplicate from each of the four locations. The results of these analyses are summarised in Table 12.

The high oleic acid soybean lines exhibit slightly elevated lectin levels when compared to the control. The values reported however are well within the literature reported range for soybeans. As lectins are readily degraded upon heating, and the levels reported are still within the literature reported range, the slightly elevated levels do not represent a safety concern.

Key anti-nutrients

Soybeans contain two well-described anti-nutritional factors. These are trypsin inhibitors and phytic acid. Trypsins inhibitors are heat labile anti-nutrients which interfere with the digestion of proteins and result in decreased animal growth. Because they are heat labile, however, they are destroyed during the processing of soy products by heat treatment. Phytic acid, on the other hand, remains stable through most soybean processing steps and has been implicated in interfering with the bioavailability of minerals such as calcium, magnesium and zinc.

Seed representing the R6 generation of lines G94-1, G94-19 and G168 were analysed for trypsin inhibitor and phytic acid content. The results are summarised in Table 13.

No significant differences were observed between the parental soybean line and the high oleic acid soybean lines for either of the anti-nutrients. The values reported are comparable to the literature reported ranges.

Other constituents

The fermentable galacto-oligosaccharides, raffinose and stacchyose, are present in soybeans and can be responsible for the production of unpleasant side effects, such as flatulence, when soybeans and soybean products are ingested. The processing of soybean flours into concentrates and isolates removes these oligosaccharides. Seeds representing the R4 and R5 generations of lines G94-1 and G94-19 were analysed for raffinose and stacchyose content. The results of the analyses are summarised in Table 14.

No significant differences were observed between the parental soybean line and the high oleic acid soybean lines for stacchyose and raffinose content. The values reported are comparable to the literature reported ranges.

Summary of the compositional analysis

The high oleic acid soybean lines exhibit slightly elevated lectin levels when compared to the control but these levels are well within the literature reported range for soybeans. As lectins are readily degraded upon heating and there are no human food uses for raw soybeans, the slightly elevated levels observed are not a cause for concern. No differences were seen in the levels of the anti-nutrients.

Analysis of the levels of various macro- and micronutrients confirmed that the high oleic acid soybeans are significantly changed with respect to their fatty acid profile. The mean oleic acid content has been increased from 23.1% in the parental soybean to 83.8% in the high oleic acid soybean lines and the linoleic acid content has been concomitantly decreased from a mean level of 55.4% to a mean level of 2.2%. Small reductions

in the levels of palmitic and linolenic acid were also observed. High oleic acid levels are found in other commonly consumed premium edible oils (*e.g.*, olive oil and high oleic acid sunflower and canola oil). The consumption of high levels of oleic acid is not considered to pose any safety concerns.

The compositional analyses revealed the unexpected occurrence of trace amounts (less than 1%) of an isomer of linoleic acid in the high oleic acid soybeans. This isomer is not present in the parental soybean line but is normally found in commonly consumed foods such as hydrogenated soybean oils and butterfat. It is present at levels in the high oleic acid soybeans that are comparable to the levels found in hydrogenated soybean oils and butterfat. Its presence is not considered to pose any toxicological or nutritional concerns.

In all other respects, the high oleic acid soybeans were found to be compositionally equivalent to the parental soybean line and other commercial varieties of soybeans.

Endogenous allergenic proteins

A separate part of the comparative analysis also considered the seed storage proteins of soybeans, which comprise a number of naturally occurring allergens. Although no new proteins are expressed in any of the high oleic acid soybean lines, they were found to exhibit a slightly altered seed storage protein profile and so a study was done to determine whether alterations to the protein profile of the high oleic acid soybeans had changed their allergenicity relative to the parental soybean line (A2396).

Soybean 7S and 11S globulins are two major storage proteins accounting for about 70% of total meal protein. The 7S fraction is made up of the α, α^1, and β subunits of β-conglycinin. The 11S fraction is made up of the acidic (A) and basic (B) subunits of glycinin. The high oleic acid soybeans were found to have reduced concentrations of the α and α^{-1} subunits of β-conglycinin, when compared with the parental A2396 soybean lines. This was coincident with an increase in the concentration of the A and B subunits of glycinin in addition to an increase in the concentration of the A2B1A glycinin precursor. The profile of other storage proteins appears to be identical to that of A2396.

The applicant speculates that the reduction in concentration of the β-conglycinin α and α^1 subunits is due to co-suppression by the α^1 promoter sequence used in the GmFad 2-1 vector (pBS43). The phenomenon of co-suppression has been observed for other genes and plants and is well documented in the literature (Brusslan and Tobin, 1995).

Radioallergosorbent (RAST) reactivity

Extracts were made of the parental soybean line A2396 and high oleic acid soybean line G94-1. Sera were used from 31 subjects with a history of documented soybean or food allergy, a positive skin test to soybean extract, and/or a positive IgE antibody response to soybean extract. Control sera were obtained from soybean tolerant individuals with a negative skin test and/or RAST to soy extract with total IgE levels similar to those sera of soybean-sensitive subjects.

In RAST reactivity assays many of the sera demonstrated significant IgE antibody reactivity to soybean extracts. Twenty-one of the 31 sera tested had IgE antibody % binding greater than or equal to 4 %. Eleven of the 21 positive sera had IgE antibody binding in excess of 20%. The sera with the most significant RAST reactivity were pooled for RAST inhibition studies.

RAST inhibition

Both the parental and high oleic acid soybean extracts yielded virtually identical RAST inhibition curves to the parental soybean RAST.

Immunoblot analysis

The 21 most potent RAST positive sera were selected for immunoblot analyses of soybean allergens. The immunoblot analysis showed, as expected, that there are a number of proteins in the soybean extract that bind IgE antibodies from soybean allergic sera. Some sera were more reactive than others, so six of the most reactive sera were selected and pooled for further study of the allergens present in the parental and high oleic acid soybeans. Both colourimetric and chemiluminescence techniques were used for the detection of reactive protein bands.

No significant differences were observed in the number of protein bands to which the sera react or to the intensity of the IgE reactivity.

Conclusion

The altered protein profile in the high oleic acid soybeans does not give rise to any significant differences in their allergen content compared to the parental soybean line A2396. Nor did the altered protein profile lead to significant changes to the total protein content of the high oleic acid soybeans.

Table 15. Effect of soybean meal varieties and processing temperature on pig F/G ratios

	Day 0 to7	*Day 7 to 14*	*Day 14 to 17*	*Day 0 to 17*
Commercial meal				
1.3% lysine	1.44	1.49	1.69	1.50
0.95% lysine	1.71	1.74	1.92	1.75
High oleic acid meal (0.95% lys)				
80-85 °C	2.38	2.42	3.56	2.49
85-90 °C	1.72	1.84	1.96	1.80
90-95 °C	1.84	1.74	1.83	1.78
100-105 °C	1.79	1.86	1.86	1.83
Check-line meal (0.95% lys)				
80-85 °C	1.75	1.86	2.03	1.84
85-90 °C	1.92	1.79	1.86	1.83
90-95 °C	1.82	1.82	1.87	1.81
100-105 °C	1.95	1.80	2.28	1.91

Nutritional impact

In assessing the safety and suitability of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

To date, all approved GM plants with modified agronomic production traits (*e.g.*, herbicide tolerance) have been shown to be compositionally equivalent to their conventional counterparts. Animal feeding studies with feeds derived from the approved GM plants have shown equivalent animal nutritional performance to that observed with the non-GM feed. Thus the evidence to date is that where GM varieties have been shown to be compositionally equivalent to conventional varieties, feeding studies using target livestock species will add little to a safety assessment and generally are not warranted (OECD 2003).

For plants engineered with the intention of significantly changing their composition or nutrient bioavailability and thus their nutritional characteristics, however, it is recognised that suitable comparators may not be available for a nutritional assessment based solely on compositional analysis. In such cases, feeding trials with one or more target species may be useful to demonstrate wholesomeness in the test animals.

In the case of the high oleic acid soybeans, significant compositional changes have been deliberately introduced into the food. The applicant therefore provided two animal feeding studies to compare the wholesomeness of the high oleic acid soybeans to controls and also undertook a study to estimate the human nutritional impact of high oleic acid soybean oil in the diet.

Animal feeding studies

Pig feeding study

This study was done to determine if soybean meal produced from high oleic acid soybeans would provide similar levels of growth performance in pigs as soybean meal from traditional varieties.

Three hundred and ninety (39/group) high-lean growth pigs (Newsham Hybrids) were fed diets consisting of processed soybean meal from either the high oleic acid soybean lines or a standard check-line soybean. The soybeans used to make the meal were processed at four different temperature ranges (80-85, 85-90, 90-95, 100-105 °C) under conditions that simulated commercial processing. Positive and negative control diets were made using commercially available soybean meal (46.5% crude protein). The positive control diet was formulated to contain dietary 1.3% lysine whereas the negative control diet was formulated to contain 0.95% dietary lysine. All test diets also contained 0.95% lysine so that any differences in growth performance could be readily attributable to the processing temperature or the amino acid availability. All pigs were fed a common 3 stage diet series until being placed on the test diets at 21 days post weaning. All test diets were corn-soybean meal based and were fed until 38 days post weaning.

Growth performance of the pigs is indicated by the average daily gain (ADG) as well as the F/G ratio, which

Table 16. Effects of processing temperature and soybean meal source on chick performance

	Daily gain 0-18 d (g)	*Feed intake 0-18 d (g)*	*Feed:gain 0-18 d (g)*	*Body weight 0-7 d (g)*	*Body weight 0-18 d (g)*
Raw					
Commercial	26.95	37.86	1.417	148.2	525.1
High oleic	15.35	30.25	1.953	101.8	316.3
Check-line	17.57	33.28	1.897	111.4	356.2
80-85 °C					
High oleic	23.60	36.66	1.570	129.6	464.8
Check-line	23.85	38.19	1.598	134.7	469.3
85-90 °C					
High oleic	24.96	38.83	1.558	136.5	489.3
Check-line	22.51	34.96	1.561	129.5	445.1
90-95 °C					
High oleic	25.71	39.53	1.540	1.45.4	502.7
Check-line	23.66	36.95	1.564	126.8	465.9
100-105 °C					
High oleic	24.03	39.07	1.628	135.0	472.5
Check-line	22.40	35.89	1.604	122.4	443.3

is a measure of the amount of the feed consumed (the average daily feed intake - ADFI) / ADG or, in other words, is an indication of how much food (in pounds) it takes to put on 1 lb of body weight in the animal. The F/G ratios obtained over the course of the study are provided in Table 15.

Pigs fed the positive control diet (commercially available soybean meal formulated to contain 1.3% dietary lysine) had increased performance (as measured by the ADG and the F/G ratio) than pigs fed any other treatment. This indicates that a dietary lysine content of 0.95% was insufficient to maximise growth performance of the pigs.

Pigs fed diets containing high oleic acid soybean meal were shown to have a similar growth performance compared to pigs fed diets containing either commercial soybean meal or meal derived from the check-line soybean formulated to similar lysine levels, when the high oleic acid soybean meal is processed at temperatures above 80-85 °C. The reason for the decreased performance, compared to the control, of pigs fed the high oleic acid soybeans processed at 80-85 °C is not readily apparent. The applicant speculates that the difference may be due to difficulties experienced with the processing of the soybeans in the pilot processing plant.

Chicken feeding study

This study was done to determine the effects of five different processing temperatures on the feeding value of the parental soybean line compared to the high oleic acid soybean lines.

Six hundred and sixteen (56/group) 1-day-old broiler chicks (Peterson x Arbor Acre) were randomly allotted to one of 11 dietary treatments. The chicks were fed diets consisting of soybean meal obtained from either a standard check-line soybean or the high oleic acid soybean lines and which had been processed at five different processing temperatures (raw, 80-85, 85-90, 90-95, and 100-105 °C). A positive control diet was included using commercially obtained high protein soybean meal. Test diets using the check-line soybean meal or the high oleic acid soybean meal were formulated to meet all nutrient requirements except for the amino acid concentration. The positive control diet contained 23% crude protein and 1.2% lysine, while diets containing check-line or high oleic acid soybean meal contained 20% crude protein and 1.03% lysine. Growth performance was measured by daily weight gain, the feed conversion ratio (feed:gain), and final body weight. The results are summarised in Table 16.

The results show that birds fed the 1.2% lysine diets (commercial soybean meal) performed significantly better in terms of their daily weight gain, feed conversion (feed:gain) and final body weight when compared to the test diets. This result is most likely attributable to the lower amino acid content of the test diets, although may also be due to differences in processing.

Table 17. The effect of replacing all oils and fats used in the domestic and commercial frying with high oleic acid soybean oil (values are means ± standard deviations)

% energy from	*High oleic acid soybean oil usage*		
	Current diet[1]	*Scenario I*	*Scenario II*
Saturated fatty acids	17.24 ± 3.44	16.61 ± 3.44	16.43 ± 3.43
Monounsaturated fatty acids	12.63 ± 2.15	14.97 ± 2.98	14.68 ± 2.86
n-3 polyunsaturated fatty acids	0.78 ± 0.27	0.73 ± 0.23	0.78 ± 0.23
n-6 polyunsaturated fatty acids	5.51 ± 2.15	3.89 ± 1.98	4.33 ± 1.92
Trans unsaturated fatty acids	2.24 ± 0.83	2.15 ± 0.83	2.12 ± 0.83

[1] *No high oleic acid soybean oil usage.*

No significant differences in performance, in either the daily weight gain or the feed conversion, between the parental soybean line and the high oleic acid soybean line were observed.

Conclusion

Interpretation of both feeding studies is complicated by the fact that they were designed to look at the effect of a number of different parameters, other than soybean variety, on feeding performance (*e.g.*, lysine content, processing temperature). Nevertheless, both demonstrate that the high oleic acid soybeans are equivalent to the commercial varieties of soybean in their ability to support typical growth and well-being in pigs and chickens.

Human nutritional impact

To assess the nutritional impact of high oleic acid soybean oil the applicant commissioned a study on the effect of high oleic acid soybean oil on the balance of dietary fats in the human diet using dietary and nutritional survey data for British adults.

The fatty acid composition of high oleic acid soybean oil was compared with those of commercial shortenings and frying oils sourced from Europe and the United States. The key findings of these comparisons were:

- The level of saturated fatty acids in high oleic acid soybean oil is similar to that in non-hydrogenated or lightly hydrogenated oils and is considerably lower than most European shortenings;
- Compared with frying oils with comparable levels of monounsaturated fatty acids, high oleic acid soybean oil has higher levels of n-6 polyunsaturated fatty acids (primarily linoleic acid);
- High oleic acid soybean oil is comparable with other frying oils for n-3 polyunsaturated fatty acids (primarily linolenic acid);
- High oleic acid soybean oil does not contain any of the trans isomers of unsaturated fatty acids found in many commercial shortenings.

For the dietary analysis two scenarios were modelled on the assumption that high oleic acid soybean oil replaced all oils present in savoury snacks, fried potatoes including chips and vegetables. It also assumed that frying oil accounted for 17% of the fat in all fried meat, eggs and fish. Because the composition of endogenous fat in the fried animal foods was not known, it had to be estimated for each food by difference between total fatty acids and a frying oil of known composition. In scenario I, a worst-case scenario, all the oil used for frying meat, eggs and fish was assumed to be a high n-6 polyunsaturated fatty acid (52.8%) corn oil. In scenario II, a more realistic scenario, the oil was assumed to be a palmolein/rapeseed (80:20) blend (12.3 % n-6 polyunsaturated fatty acids). Assumptions also had to be made about the level of n-6 polyunsaturated fatty acids in high oleic acid soybean oil as this level can be influenced by crop growth conditions. Commercially available high oleic acid soybean oil is anticipated to contain 2.2% n-6 polyunsaturated fatty acids but batches as low as 0.9% have been observed under certain field conditions. A n-6 polyunsaturated fatty acid content of 0.9% for high oleic acid soybean oil was assumed for scenario I and 2.2% was assumed for scenario II.

A summary of the main findings of the analysis is presented in Table 17.

The analysis shows that the impact of the high oleic acid soybean oil use on the intakes of saturated fatty acids is quite small, equivalent to a 5% reduction at best, with little difference between the two scenarios. The intake of monounsaturated fatty acids would increase at best by 19%, with again little difference between the two scenarios. The intake of n-6 polyunsaturated fatty acids would fall by 29% for scenario I and by 21% for scenario II. The analysis also

Table 18. A comparison of the effect of replacing all oils and fats used in frying and in the manufacture of savoury snacks with either high oleic acid soybean oil or olive oil (values are means)

Oil	*% energy from*				
	Scenario	*Mono*	*n-6 poly*	*n-3 poly*	*Saturated*
High oleic	I	15.7	3.2	0.8	16.6
Olive	I	15.6	3.3	0.7	16.7
High oleic	II	15.1	4.2	0.8	16.1
Olive	II	15.0	4.3	0.8	16.2
Current UK diet		**12.6**	**5.5**	**0.8**	**17.2**

Table 19. A comparison of mean percentage energy from fatty acids in British and Australian diets

Country	*Mean % Energy from fatty acid type*		
	Mono	*Poly*	*Saturated*
United Kingdom	12.6	6.3	17.2
Australia	11.8	5.0	12.7

shows that there would be little or no change to the intakes of n-3 polyunsaturated fatty acids or trans unsaturated fatty acids with either scenario.

To put the use of high oleic acid soybean oil into context, the analysis was repeated using a low n-6 olive oil (79.3% monounsaturated fatty acids, 0.7% n-3 polyunsaturated fatty acids and 6% n-6 polyunsaturated fatty acids) to replace all of the fats and oils considered in the analysis. The results of this analysis are presented in Table 18.

This analysis shows that, were low n-6 olive oil to replace all the fats considered in the analysis, the impact would be very similar to that of high oleic acid soybean oil under similar conditions.

The study concluded that while the use of high oleic acid soybean oil might lower dietary linoleic acid intake somewhat (by an absolute maximum of 29%), it would not do so to any level that would be a public health concern in terms of cardiovascular disease. Moreover, it was concluded that such a reduction could apply equally to many existing commercially available low n-6 polyunsaturated frying oils, such as olive oil.

Therefore, the overall finding of the study was that the nutritional impact of the use of high oleic acid soybean oil as a replacement for frying fats was likely to be beneficial because diets incorporating high oleic acid soybean oil show decreased saturated fatty acid intakes and this is likely to reduce risk factors for cardiovascular disease.

The general conclusion of this report were then applied to the Australian context and indicate that the magnitude of the changes is likely to be reduced. Table 19 shows a comparison of the fatty acid profiles of the United Kingdom and Australia from recent national dietary surveys.

The fall in mean polyunsaturated intakes quoted for the British case above assumes 100% replacement. In reality, this is unlikely to happen, and data given in the report show that, with successive reductions in the % replacement, intakes progressively increase towards original levels. For example at 25% percent replacement, percentage energy from PUFA decreases to 6.0%.

There are some high monounsaturated oils available or soon to be available on the Australian market that have been created through conventional plant breeding and selection techniques from sunflower and rapeseed stock. These types of oils have been successful in replacing a proportion of palm oil mixes in food manufacture and retail frying. Olive oil has also become a popular oil for domestic use.

Conclusions

The information summarised in this case study was used for safety assessment in Australia and New Zealand.

FSANZ stated the following as a summary of their evaluation of the high oleic acid soybeans:

Three lines of a new variety of soybean (G94-1, G94-19 and G168), high in the monounsaturated fatty acid oleic acid, were generated by the transfer of a second copy of a soybean fatty acid desaturase gene (GmFad 2-1) to a high yielding commercial variety of

soybean (line A2396). The fatty acid desaturase is responsible for the synthesis of linoleic acid, which is the major polyunsaturated fatty acid present in soybean oil. The presence of a second copy of the fatty acid desaturase gene causes a phenomenon known as "gene silencing" which results in both copies of the fatty acid desaturase gene being "switched off", thus preventing linoleic acid from being synthesised and leading to the accumulation of oleic acid in the developing soybean seed.

Soybeans are grown as a commercial crop in over 35 countries worldwide and have a long history of safe use as human food. The major food product to be derived from the high oleic acid soybeans will be the oil. High oleic acid soybean oil will be predominantly used in spraying and frying applications and might replace heat stable fats and oils such as hydrogenated soybean and rapeseed oil or palm olein/vegetable oil blends.

Other genes transferred along with the GmFad 2-1 gene were the uidA gene and the bla gene. The uidA gene is a colourimetric marker used for selection of transformed plant lines during the soybean transformation procedure. It codes for the enzyme β-glucuronidase and is derived from the bacterium *Escherichia coli*. The bla gene is a marker used to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells. It codes for the enzyme β-lactamase and confers resistance to some β-lactam antibiotics, such as penicillin and ampicillin. The use of the bla gene as a selectable marker was not considered to pose any safety concerns.

The transferred genes were all found to be stably integrated into the genome of the high oleic acid soybean lines and are all phenotypically and genetically stable over multiple generations and in various environments.

Extensive analyses of the high oleic acid soybeans demonstrated that none of the transferred genes give rise to a protein product, meaning no new proteins are expressed in any of the high oleic acid soybean lines.

The composition of the high oleic acid soybeans was compared to that of the elite soybean line from which they were derived. These comparisons examined the key nutrients, toxicants and anti-nutrients of soybeans, as well as the protein profile.

Soybeans contain the toxicant lectin as well as the anti-nutrients trypsin inhibitor and phytate. The high oleic acid soybean lines exhibit slightly elevated lectin levels when compared to the control but these levels are well within the literature reported range for soybeans. As lectins are readily degraded upon heating and there are no human food uses for raw soybeans, the slightly elevated levels observed are not a cause for concern. No differences were seen in the levels of the anti-nutrients.

Comparisons were also made with the levels of various macro- and micronutrients. Proximate (crude fat/protein, fibre, ash), amino acid, fatty acid, vitamin and mineral, and isoflavone levels were measured. These analyses confirmed that the high oleic acid soybeans are significantly changed with respect to their fatty acid profile. The mean oleic acid content has been increased from 23.1% in the parental soybean to 83.8% in the high oleic acid soybean lines and the linoleic acid content has been concomitantly decreased from a mean level of 55.4% to a mean level of 2.2%. Small reductions in the levels of palmitic and linolenic acid were also observed. High oleic acid levels are found in other commonly consumed premium edible oils (*e.g.*, olive oil and high oleic acid sunflower and canola oil). The consumption of high levels of oleic acid is not considered to pose any safety concerns.

The compositional analyses revealed the unexpected occurrence of trace amounts (less than 1%) of an isomer of linoleic acid in the high oleic acid soybeans. This isomer is not present in the parental soybean line but is normally found in commonly consumed foods such as hydrogenated soybean oils and butterfat. It is present at levels in the high oleic acid soybeans that are comparable to the levels found in hydrogenated soybean oils and butterfat. Its presence is not considered to pose any toxicological or nutritional concerns.

The seed storage proteins of soybeans, which comprise a number of naturally occurring allergens were also compared. Although no new proteins are expressed in any of the high oleic acid soybean lines, they were found to exhibit a slightly altered seed storage protein profile. Allergenicity testing confirmed, however, that the altered protein profile does not give rise to any significant differences between the allergen content of the high oleic acid soybeans and the parental soybean line A2396. Nor did the altered protein profile lead to significant changes to the total protein content of the high oleic acid soybeans.

In all other respects, the high oleic acid soybeans were found to be compositionally equivalent to the parental soybean line and other commercial varieties of soybean.

Two animal feeding studies, with pigs and chickens, were done with the high oleic acid soybeans.

These studies confirmed that the high oleic acid soybeans are equivalent to other commercial varieties of soybean with respect to its ability to support typical growth and well-being.

A study was also undertaken to assess the human nutritional impact of the use of high oleic acid soybean oil as a replacement for frying fats. The study concluded that the use of high oleic acid soybean oil might lower dietary linoleic acid intake somewhat (by an absolute maximum of 29%), but it would not do so to any level that would be a public health concern in terms of cardiovascular disease. Overall, the conclusion of the study was that the nutritional impact of the use of high oleic acid soybean oil was likely to be beneficial because diets incorporating high oleic acid soybean oil show decreased saturated fatty acid intakes and this is likely to reduce risk factors for cardiovascular disease.

Overall it was concluded that the high oleic acid soybeans are significantly changed with respect to their fatty acid profile but are comparable to non-GM soybeans in terms of their safety and nutritional adequacy.

On the basis of this safety assessment, food from high oleic soybean lines G94-1, G94-19 and G168 was approved in Australia and New Zealand in November 2000.

References

Astwood, J.D., Leach, J.N. and Fuchs, R.L. (1996). Stability of food allergens to digestion *in vitro*. *Nature Biotechnology* 14: 1269-1273.

Barker, S.J., Harada, J.J. and Goldberg, R.B. (1988). *Proc. Natl. Acad. Sci.* (USA) 85: 458-462.

Berry-Lowe, S.L., McKnight, T.D., Shah, D.M. and Meagher, R.B. (1982). *J. Mol. Genet.* 1: 483-498.

Bevan, M., Barnes, W.M. and Chilton, M. (1983). Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res.* 11: 369-385.

Bonnassie, S., Oreglia, J. and Sicard, A.M. (1990). Nucleotide sequence of the *dapA* gene from Corynebacterium glutamicum. *Nucleic Acids Res.* 18: 6421.

Brock, T.D., Smith, D.W. and Madigan, M.T. (1984). *The Biology of Microorganisms, 4th Edition*. Prentice Hall International Inc, New Jersey, 847 pp.

Brusslan, J.A., and Tobin, E.M. (1995). Isolation of new promoter-mediated co-suppressed lines in *Arabidopsis thaliana*. *Plant Molec. Biol.* 27: 809-813.

Calva, J.J, Sifuentes-Osbornio, J. and Ceron, C. (1996). Antimicrobial resistance in fecal flora: longitudinal community-based surveillance of children from urban Mexico. *Antimicrobial Agents and Chemotherapy* 40: 1699-1701.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.M. (1982). Nopaline synthase: transcript mapping and DNA sequence. *J. Molec. Appl. Genet.* 1: 561-573.

Doyle, J.J., Schuler, M.A., Godette, W.D., Zenger, V., Beachy, R.N. and Slightom, J.L. (1986). The glycosylated seed storage proteins of *Glycine max* and *Phaseolis vulgaris*. Structural homologies of genes and proteins. *J. Biol. Chem.* 261: 9228-9238.

Falco, S.C., Guida, T., Locke, M., Mauvais, J., Sanders, C., Ward, R.T., Weber, P. (1995). *Bio/Technology* 13: 577-582.

Harpster, M.H. *et al.* (1989). *Mol. Gen. Genet.* 212: 182-190.

Heppard, E.P., Kinney, A.J., Stecca, K.L., Miao, G-H (1996). Developmental and growth temperature regulation of two different microsomal omega-6 desaturase genes in soybeans. *Plant Physiol.* 110: 311-319.

Ingelbrecht, I., Van Houdt, H., Van Montagu, M. and Depicker, A. (1994). Post-transciptional silencing of reporter transgenes in tobacco correlates with DNA methylation. *Proc. Natl. Acad. Sci.* (USA) 91: 10502-10506.

Jefferson, R.A., Burgess, S.M., and Hirsh, D. (1986). β-glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci.* (USA) 83: 8447-8451.

Jofuku, D. and Goldberg, R.B. (1989). Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. *Plant Cell* 1: 1079-1093.

Jones, D.D. and Maryanski, J.H. (1991). Safety considerations in the evaluation of transgenic plants for human food. In: Levin MA and Strauss HS (eds) Risk assessment in genetic engineering. New York: McGraw-Hill.

Kilby, N.J., Ottoline Leyser, H.M. and Furner, I.J. (1992). Promoter methylation and progressive transgene inactivation in *Arabidopsis. Plant Mol. Biol.* 20: 103-112.

Kinney, A.J. (1994). *Curr. Opin. Biotechnol.* 5: 144-151.

Lehrer, S.B. and Reese, G. (1998). Food allergens: implications for biotechnology. In: Thomas JA (ed.) Biotechnology and safety assessment. Taylor and Francis, Philadelphia.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. and Fuchs, R.L. (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Food Sci. Nut.* 36(S): S165-S186.

Neu, H.C. (1992). The crisis in antibiotic resistance. *Science* 257: 1064-1073.

Odell, J.T., Nagy, F., and Chua, N-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313: 810-812.

OECD (2001). *Consensus document on compositional considerations for new varieties of soybean: key food and feed nutrients and anti-nutrients*. Series on the Safety of Novel Foods and Feeds, No. 2. Organisation of Economic Cooperation and Development, Paris.

OECD (2003). *Considerations for the safety assessment of animal feedstuffs derived from genetically modified plants*. Series on the Safety of Novel Foods and Feeds, No. 9. Organisation of Economic Cooperation and Development, Paris.

Okuley, J., Lightner, J., Feldman, K., Yadav, N., Lark, E. and Browse, J. (1994). *Arabidopsis* FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell* 6: 147-158.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual, 2nd Edition*. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Sjoblad, R.D., McClintock, J.T. and Engler, R. (1992) Toxicological considerations for protein components of biological pesticide products. *Regulatory Toxicol*. Pharmacol. 15: 3-9.

Voinnet, O. (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. *Current Opinion in Plant Biology* 5:444-451

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature* 406: 775-781.

Wang, M. and Waterhouse, P.M. (2001) Application of gene silencing in plants. *Current Opinion in Plant Biology* 5:146-150

WHO (1993) *Health aspects of marker genes in genetically modified plants*. Report of a WHO Workshop. World Health Organization, Geneva.

WHO (2000). *Safety aspects of genetically modified foods of plant origin*. Report of a Joint FAO/WHO Expert Consultation on foods derived from biotechnology. World Health Organization, Geneva.

Yadav, N.S., Wierzibicki, A., Aegerter, M., Caster, C.S., Perez-Grau, L., Kinney, A.J., Hitz, W.D. *et al.* (1993). Cloning of higher plant omega-3 fatty acid desaturases. *Plant Physiol.* 103: 467-476.

Yeh, P., Sicard, A.M. and Sinskey, A.J. (1988). *Mol. Gen. Genet.* 212: 105-111 ●

Case study 3

Food safety assessment of a genetically modified herbicide tolerant soybean

Preface

This teaching module has been developed as a tool for providing regulators with practical training in GM food safety assessment. The specific safety assessment approach discussed in this text is based on the Canadian regulatory framework for biotechnology products and on Health Canada policy. Nonetheless, the concepts are consistent with those described in international consensus documents produced by the Organization for Economic Cooperation and Development (OECD), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations.

In order to provide some insight into the type of data usually presented in support of a GM food evaluation, a case study of genetically engineered soybean (Glycine max) event GTS 40-3-2 and its progeny has been developed. The content of the study includes excerpts from applications for food safety assessment submitted to regulatory authorities in Canada, the United Kingdom (UK), and the United States (US).

A note on quality standards for documentation

The evaluation of an application for a GM food safety assessment is comparable to the peer review of a manuscript for publication in a scientific journal. Accordingly, the quality of the text and data presented must be commensurate with this. Experimental procedures should be described in sufficient detail (or referenced accordingly) so that the methodology can be repeated. Spelling and usage should be standard and laboratory jargon avoided. It is recommended that international standards for nomenclature be adopted, such as those described in the International Union of Biochemistry and Molecular Biology's Biochemical Nomenclature and Related Documents [(1992) 2nd Ed. Portland Press, Inc., Chapel Hill, NC], which contains the International Union of Biochemistry rules of nomenclature for amino acids, peptides, nucleic acids, polynucleotides, vitamins, co-enzymes, quinones, folic acid and related compounds, corrinoids, lipids, enzymes, proteins, cyclitols, steroids, carbohydrates, carotenoids, peptide hormones, and human immunoglobulins. Correct chemical names should be given and strains of organisms should be specified. Trade names should be identified. Système International (SI) units and symbols should be used whenever possible.

Illustrations, tables and figures must be clear and legible. Original drawings, high-quality photographs or laser prints are acceptable; poor-quality reproductions that often result from photocopying prints are not. In particular, reproductions of gels or blots must be of sufficient quality to clearly show the described results.

Disclaimer

Monsanto Inc. has generously consented to the use of the information provided in various of their regulatory submissions for event GTS 40-3-2 as a training tool. It must be noted, however, that in order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original applications. Certain information has been reduced to summaries and the data as presented in the case study are only a subset of that actually submitted. The case study in no way constitutes a complete application nor is it to be considered a complete safety assessment. To that end, the use of this information in the form of a training tool does not constitute an endorsement of the information or product nor should it be considered a reflection of any of the original submissions.

Description of the recombinant-DNA plant

Soybean is grown as a commercial crop in over 80 countries, with a combined harvest of 162 million metric tonnes. The major producers of soybeans in 2000 were the United States, Brazil, China, Argentina, India, Canada and Paraguay. Soybean is grown primarily for its seed, which has many uses in the food and industrial sectors, representing one of the major sources of edible vegetable oil and of proteins for livestock feed use.

A major food use of soybean in North America and Europe is as purified oil, used in margarines, shortenings, and cooking and salad oils. It is also a major ingredient in food products such as tofu, tempeh, soya sauce, simulated milk and meat products, and is a minor ingredient in many processed foods. Soybean meal is used as a supplement in feed rations for livestock.

Weeds are a major production problem in soybean cultivation. Typically, weeds are managed using a combination of cultural (*e.g.* seed bed preparation, using clean seed, variety selection, and planting date) and chemical controls. Depending on the production

area and the prevalent weed species, herbicides may be applied before planting (*e.g.* pendimethalin, trifluralin, metribuzin), after planting but before emergence (*e.g.* pendimethalin, linuron, imazethapyr), and/or after emergence (*e.g.* bentazon, acifluorfen, fomesafen). Commonly, several different herbicides are required to adequately control weeds in soybean fields.

The soybean line GTS 40-3-2 was developed to allow for the use of glyphosate, the active ingredient in the herbicide Roundup®, as a weed control option. This genetically engineered soybean line contains a form of the plant enzyme 5- enolpyruvylshikimate-3-phosphate synthase (EPSPS) that allows GTS 40-3-2 to survive the otherwise lethal application of glyphosate. The EPSPS gene put into GTS 40-3-2 was isolated from a strain of the common soil bacterium *Agrobacterium tumefaciens* called CP4; the form of EPSPS enzyme produced by this gene is tolerant to glyphosate.

The EPSPS enzyme is part of an important biochemical pathway in plants called the shikimate pathway, which is involved in the production of aromatic amino acids and other aromatic compounds. When conventional plants are treated with glyphosate, the plants cannot produce the aromatic amino acids needed to grow and survive. EPSPS is present in all plants, bacteria, and fungi. It is not present in animals, which do not synthesize their own aromatic amino acids. As the aromatic amino acid biosynthetic pathway is not present in mammals, birds or aquatic life forms, glyphosate has little if any toxicity for these organisms. The EPSPS enzyme is naturally present in foods derived from plant and microbial sources.

GTS 40-3-2 was developed by introducing the CP4 EPSPS gene into a commercial soybean variety using particle-acceleration (biolistic) transformation. The glyphosate tolerance trait expressed in GTS 40-3-2 has since been transferred into more than one thousand commercial soybean varieties by traditional breeding techniques.

GTS 40-3-2 has been tested in field trials in the United States, Central and South America, Europe, and Canada since 1991. Data collected from over 150 field trials conducted over a three-year period prior to commercialization in the United States demonstrated that GTS 40-3-2 did not differ significantly from conventional soybeans in morphology, seed production (yield), agronomic characteristics (such as time to flowering and pod set, or vigor) and tendency to weediness. GTS 40-3-2 did not negatively affect beneficial or nontarget organisms, and was not expected to impact on threatened or endangered species.

Table 1. Regulatory approval status of glyphosate tolerant soybean event GTS-40-3-2

Country	*Environment (year)*	*Food and/or feed (year)*	*Marketing (year)*
Argentina	1996	1996	
Australia		2000	
Brazil	1998	1998	
Canada	1995	1996	
China		2004	
Czech Republic		2001	2001
European Union			1996
Japan	1996	1996	
Korea		2000	
Mexico	1998	1998	
Philippines		2003	
Russia		1999	1999
South Africa	2001	2001	
Switzerland		1996	
Taiwan		2002	
United Kingdom		1996	
United States	1994	1994	
Uruguay	1997	1997	

Soybean does not have any weedy relatives with which it can crossbreed in the continental United States or Canada. Cultivated soybean can naturally cross with the wild annual species G. *soja*, however G. *soja*, which occurs naturally in China, Korea, Japan, Taiwan and the former USSR, is not naturalized in North America. Additionally, soybean plants are almost completely self-pollinated and reproductive characteristics such as pollen production and viability were unchanged by the genetic modification resulting in GTS 40-3-2. It was therefore concluded that the potential for transfer of the glyphosate tolerance trait from the transgenic line to soybean relatives through gene flow (outcrossing) was negligible in managed ecosystems, and that there was no potential for transfer to wild species in Canada and the continental United States.

The food and livestock feed safety of GTS 40-3-2 soybean was established based on: the evaluation of the similarity of the structure and function of CP4 EPSPS protein to this same enzyme naturally present in foods and livestock feeds, the fact that CP4 EPSPS protein constitutes a small amount of the protein in GTS-40-3-2 soybeans so there is little dietary exposure, the lack of toxicity or allergenicity of EPSPS proteins from plants, bacteria and fungi, and by direct laboratory studies of the CP4 EPSPS protein. Comparative analyses of key nutrients, including proximates (*e.g.* protein, fat, fibre,

ash, and carbohydrates), as well as antinutrients between GTS 40-3-2 soybeans and conventional soybeans did not reveal any significant differrences. Feeding studies with rats, broiler chickens, cows, and fish further supported the safety and nutritional quality of GTS 40-3-2 as human food and livestock feed.

Event GTS 40-3-2 received its first regulatory approval in the US in 1994 (US Department of Agriculture), and has since been approved for environmental release and use in livestock feed and/or human food in 17 countries and the European Union (Table 1). In 1996, glyphosate tolerant soybeans were planted on less than 5% of the US soybean acreage. In the 2000 growing season, 54% of the soybeans – approximately 40 million acres of the 75.4 million acres of soybeans grown in the United States – were glyphosate tolerant. In Argentina, where the adoption rate is estimated at 95%, glyphosate tolerant soybeans were grown on over 20 million acres in 2000. Globally, glyphosate tolerant soybeans made up 58% of all transgenic crops grown in 2000.

Description of the host plant and its use as food

The genus *Glycine* Willd. is a member of the family Leguminosae, subfamily Papilionoideae, and the tribe Phaseoleae. The genus *Glycine* is of Asian and Australian origin (Lackey, 1981). *Glycine* is divided into two subgenera, *Glycine* and *Soja* (Moench) F. J. Herm. The subgenus *Glycine* consists of 12 wild perennial species (Hymowitz *et al.* 1991) with wide distribution patterns: Australia, South Pacific Islands, West Central Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan (Hermann, 1962; Newell & Hymowitz, 1978; Hymotitz & Newell, 1981; Grant *et al.* 1984a, 1984b; Tindale 1984, 1986a, 1986b). The subgenus *Soja* includes the cultivated soybean, *G. max* (L.) Merrill, and its nearest wild relative, G. *soja* Sieb. and Zucc., that has been found in China, Taiwan, Japan, Korea, and the former USSR. Both of these species are annuals.

Soybean is a cultivated species of the legume family. Soybeans grow on erect, bushy annual plants, 0.3 - 1.2 metres high with hairy stems and trifoliate leaves. The flowers are small in axillary racemes, usually white or purple. The male and female floral organs are enclosed within the corolla. The seeds are produced in pods, usually containing three spherical to oval seeds weighing 0.1–0.2 g. More detailed descriptions of soybean morphology can be found in Hermann (1962) and Carlson & Lerston (1987).

Glycine is the only genus in the Phaseoleae where species have diploid chromosome numbers of 40 and 80 but not 20. The unique chromosome number of *Glycine* is probably derived from diploid ancestors with base number 11, which have undergone aneuploid loss to base number 10 (Lackey 1988). In the legumes, only 10 of 71 genera are considered completely polyploid, *Glycine* is one of these (Senn, 1938). The soybean should be regarded as a stable tetraploid with diploidized genomes (Gurley *et al.* 1979; Lee & Verrna 1984; Skorupska *et al.* 1989).

Soybean is native to China. Early Chinese history refers to soybeans in books written over 4500 years ago (Hymowitz & Singh 1987). Soybean is believed to have been domesticated in the eastern half of northern China around the 1lth century B.C. or earlier (Hymowitz 1970), and its cultivation subsequently extended throughout south-east Asia. Soybean is believed to have been introduced into Western Europe in the 18th century (Wolf 1983), though Europe today is a minor producer of soybean, producing less than 2% of the world's production (Oil World Annual 1992). Soybean was introduced into the USA in 1765 (Hymowitz & Harlan 1983), primarily as a forage crop grown for hay and silage. Successful use of soybean as an oilseed in Europe from 1900 to 1910 promoted interest in its use in the USA. Even though interest in soybean production was on the increase during the 1920s and 1930s, most soybean acres were used for forage. The first U.S cultivars selected from planned cross-pollinations were released in the 1940s. Cultivars selected from the first populations formed by hybridization were used as parents to form populations for additional cycles of selection. The process of utilizing superior progeny from one cycle of selection as parents to form populations for the next cycle continues up to the present time (Burton 1987).

In the United States, there has been a rapid expansion in the cultivation of soybean over the past fifty years. Soybean production regions in the USA are concentrated in the Midwest and in the Mississippi Valley (Hazera & Fryar 1981). Apart from the United States (59.8 million metric tons in 1992/93), the principle soybean production areas are now in Brazil (21.3 MT), Argentina (11.7 MT), the Peoples Republic of China (9.7 MT) and India (USDA 1993). The main soybean producing states in Brazil are Rio Grande Do Sul, Parana, and Mato Grosso. In Argentina, the main soybean growing areas are the provinces of Sante Fe, Buenos Aires, and Cordoba.

In Western Europe, soybean is grown mainly in Italy (0.2 -0.4 Mha), in France (0.05 -0.15 Mha), and

occasionally in Greece and Spain. French soybean production is located mainly in the south west and in the Loire valley. In Italy, the soybean production areas are located in the Po valley, particularly in the Po delta and on the coastline of the Veneto region. Europe is one of the major world importers of soybeans.

Soybean is known to contain a number of natural antinutritional components (Rackis 1974; Orthoefer 1978). Trypsin (protease) inhibitors are known to have antinutritive properties in animals fed unprocessed soybeans (Rackis 1974; Rackis *et al.* 1986), although adequate heating inactivates trypsin inhibitors. Soybean hemagglutinin is known to cause red blood cell agglutination *in vitro* (Leiner, 1953), but there is no clear evidence that soybean hemagglutinin plays an antinutritive role (Rackis 1974). The phytoestrogens genistein, daidzein and coumesterol, naturally present in soybeans, are reported to possess a number of biochemical activities in mammalian species, including estrogenic and hypocholesterolemic activities (Wang *et al.* 1990; Murphy 1982). The low molecular weight carbohydrates stachyose and raffinose are known to cause flatus activity (Rackis 1974). Phytic acid (phytate) may reduce mineral availability, since it exists in soybeans as an insoluble, non-nutritionally available calcium-magnesium-potassium complex (Orthoefer 1978; Mohamed *et al.* 1991).

Soybean is also known to be the cause of food allergies in certain individuals (Burks *et al.* 1988). Although the specific soybean proteins that elicit the allergenic reactions in soybean have not been uniquely identified or characterised, these proteins have typically been characterised by immunoblotting (Bush *et al.* 1988; Shibasaki *et al.* 1980). Using this technique, specific protein bands have been identified that react with the IgE antibody produced from a pool of sera from soybean sensitive individuals. The number of allergenic proteins varies with sera obtained from individuals in different countries, probably reflecting the extent of consumption of soybean products in the diet. Data from one study in the United States (Bush *et al.* 1988) showed 9 different allergenic proteins using the immunoblot technique, whereas a study in Japan using the same procedure (Shibasaki *et al.* 1980) concluded that there may be as many as 15 different allergenic proteins.

G. max L. cv. A5403 ("A5403"), the cultivar that was genetically modified to be tolerant to glyphosate, is a maturity group V cultivar which combines a consistently high yield potential with resistance to races 3 and 4 of the soybean cyst nematode (SCN). It has purple flowers, grey pubescence and tan pods. Seeds are dull yellow with imperfect black hila. A5403 also combines good standability, excellent emergence, and tolerance to many leaf and stem diseases. A5403 was one of the first group V cultivars with SCN resistance provided to farmers and has received protection under the United States Plant Variety Protection Act. The commercialization strategy for GTS 40-3-2 is to use traditional backcrossing and breeding to transfer the glyphosate tolerance locus from this cultivar to a wide range of varieties and maturity groups of soybeans.

Soybean has a history of safe use as food. Soybeans or processed fractions are consumed in many human food products or animal feeds; soybean is one of the world's largest sources of plant protein and oil. Consequently, the characteristics of soybean in general, and more specifically progenitor line A5403, do not warrant analytical or toxicological tests. Typically, soybean breeders make genetic crosses to generate new cultivars with enhanced commercial value, and they evaluate new varieties primarily based on yield, as well as protein and oil content.

References

Burton, J. W. (1987). Quantitative genetics: results relevant to soybean breeding, *Agronomy* 16: 211-247.

Burks, A.W., Brooks, J.R., Sampson, H.A. (1988). Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) & immunoblotting in children with atopic dermatitis and positive soy challenge. *Journal of Allergies and Clinical Immunology* 81:1135-1142.

Bush, R.K., Schroeckenstein, D., Meier-Davis, S., Balmes, J., & Rempel, D. (1988). Soybean flour asthma: detection of allergens by immunoblotting. *Journal Allergy and Clinical Immunology* 82: 251-255.

Carlson, J. B. & Lersten, N. R. (1987). Reproductive morphology. *Agronomy* 16: 95-134.

Grant, J.E., Brown, A.H.D. & Grace J.P. (1984a). Cytological and isozyme diversity in Glycine tomentella Haryata (Legumonosae). *Australian Journal of Botany* 32: 665-677.

Grant, J.E., Grace, J.P., Brown, A.H.D. & Putievsky, E. (1984b). Interspecific hybridization in Glycine Willd. Subgenus Glycine (Legumonosae). *Australian Journal of Botany* 32: 655-663.

Gurley, W.B., Hepburn, & A.G., Key, J.L. (1979). Sequence organization of the soybean genome. *Biochem. Biophys. Acta* 561: 167-183.

Hazera, J. & Fryar, E. (1981). Regional soybean production since 1960 and the outlook for the 1980s. Economic Research Service USDA F05-305. U.S. Govt. Printing Office, Washington, D.C.

Hermann, F. J. (1962). A revision of the genus Glycine and its immediate allies. United States Department of Agriculture Technical Bulletin 1268: 1-79.

Hymowitz, T. (1970). On domestication of soybean. *Economic Botany* 24: 408-421.

Hymowitz, T. & Newell, C. A. (1981). Taxonomy of the genus Glycine, domestication and uses of soybeans. *Economic Botony* 35: 272-288.

Hymowitz, T. & Harlan, J. R. (1983). Introduction of soybeans to North America by Samuel Bowen in 1765. *Economic Botany* 37: 371-379.

Hymowitz, T. & Singh, R. J. (1987). Soybean Monograph, Soybeans: Improvement, Production, and Uses. (2nd ed.). Wilcox, R. J. ed., pp. 23-48.

Hymowitz, T., Palmer, R.G. & Singh, R.J. (1991). Cytogenetics of the genus Glycine. In Chromosome Engineering in Plants: Genetics, Breeding, Evolution, Part B. Tsuchiya, T. and Gupta, P. K., eds. Elsevier Science Publishers B. V., Amsterdam, pp 53-63.

Lackey, J. A. (1988). Chromosome numbers in the Phaseoleae (Fabaceae:Faboideae) and their relation to taxonomy, *American Journal of Botany* 67: 595-602.

Lackey, J. A. (1981). Phaseoleae DC. In Advances in Legume Systematics, Part l. Pohill, R. M. and Raven, R.H. eds. Royal Botanic Gardens, Kew, pp.301-327.

Lee, J. S. & Verma., D.P.S. (1984). Structure and chromosome arrangement of leghemoglobin genes in kidney bean suggest divergence in soybean leghemoglobin gene loci following tetraploidization. *EMBO J.* 3: 2745-2752.

Leiner, I. E. (1953). Soyin, a toxic protein from the soybean. I. inhibition of rat growth. *Journal of Nutrition* 49:527-540.

Mohamed, A.I., Mebrahtu, T., & Rangappa, M. (1991). Nutrient composition and anti-nutritional factors in selected vegetable soybean [Glycine max (L.) Merr.]. *Plant Foods for Human Nutrition* 41: 89-100.

Murphy, P. A. (1982). Phytoestrogen content of processed soybean products. *Food Technology* 36: 60-64.

Newell, C. A. & Hymowitz, T. (1978). A reappraisal of the subgenus Glycine. *American Journal of Botany* 65:168-179.

Oil World Annual (1992). ISTA Mielke GmbH. Germany

Orthoefer, F. T. (1978). Processing and Utilization. In Soybean Physiology, Agronomy, and Utilization, A. G. Norman, ed. Academic Press, New York. pp. 219-246.

Rackis, J. J. (1974). Biological and physiological factors in soybeans. Journal of American Oil Chemists' Society 51:161A-173A.

Rackis, J.J., Wolf, W.J., & Baker, E.C. (1986). Protease inhibitors in plant foods: content and inactivation. In Nutritional and Toxicological Significance of Enzyme Inhibitors in Food. M. Friedman, (ed.). Plenum Press, New York. pp. 299-347.

Senn, H. A. (1938). Chromosome number relationships in the Leguminosae. *Biblio. Genet.* 12: 175-336.

Shibasaki, M., Suzuki, S., Tajima, S., Nemoto, H., & Kuroume, T. (1980). Allergenicity of major component proteins in soybean. *International Archives of Allergy and Applied Immunology* 61:441-448.

Skorupska, H., Albertsen, M.C., Langholz, K.D. & Palmer, R.G. (1989). Detection of ribosomal RNA genes in soybean, *Glycine max* (L.) Merr., by in situ hybridization. *Genome* 32: 1091-1095.

Tindale, M. D. (1984). Two new Eastern Australian species of Glycine Willd. (Fabaceae). *Brunonia* 7:207-213.

Tindale, M. D. (1986a). A new North Queensland species of Glycine Willd. (Fabaceae). *Brunonia* 9:99-103.

Tindale, M. D. (1986b). Taxonomic notes on three Australian and Norfolk Island species of Glycine Willd. (Fabaceae:Phaseolae) including the choice of a neotype of G. clandestine Wendl. *Brunonia* 9:179-191.

United States Department of Agriculture (USDA) (1993). World oilseed situation and outlook. USDA Foreign Agricultural Service, Oilseeds and Products (FOP 4-93).

Wang, G., Kuan, S.S., Francis, O.J., Ware, G.M. & Carman, A.S. (1990). A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *Journal of Agriculture and Food Chemistry* 38:185-190.

Wolf, W. J. (1983). Edible soybean protein products. In CRC Handbook of Processing and Utilization in Agriculture, Vol. II, Part 2, I. A. Wolff, (ed.). CRC Press, Boca Raton, pp. 23-55.

Description of the donor organism(s)

The donor genes

GTS 40-3-2 contains DNA sequences derived from the following donor organisms:

1. *Agrobacterium* sp. strain CP4 EPSPS gene: The C-terminal 1.36 kb 5-enolpyruvylshikimate-3-phosphate synthase gene (CP4 EPSPS) (Barry *et al.*, 1992; Padgete *et al.*, 1993).
2. Cauliflower mosaic virus (CaMV) enhanced 35 S promoter (P-E35S): The CaMV promoter (Odell *et al.* 1985) with the duplicated enhancer region (Kay *et al.*, 1985).
3. Petunia hybrida chloroplast transit peptide (CTP): The N-terminal 0.22 kb CTP sequence for the P. hybrida EPSPS gene (Shah *et al.*, 1986). The CTP sequence was fused to the N-terminus of the CP4 EPSPS gene to deliver the CP4 EPSPS protein to the chloroplast, the site of EPSPS activity and glyphosate action.
4. *Agrobacterium tumefaciens* 3' untranslated region of the nopaline synthase gene (NOS 3'): The NOS 3' sequence, isolated from the *A. tumefaciens* Ti plasmid, provides the polyadenylation signal for stable expression (Fraley et al., 1983).

None of the inserted sequences are known to have any pathogenic or harmful characteristics.

The following sequences were present on plasmid PV-GMGT04 but were not integrated into the GTS 40-3-2 genome:

1. Neomycin phosphotransferase II encoding bacterial marker gene (*nptII*): The bacterial selectable marker gene, *nptII*, isolated from the prokaryotic transposon, Tn5 (Beck *et al.*, 1982), encodes for the enzyme neomycin phosphotransferase. This enzyme confers resistance to aminoglycoside antibiotics (*e.g.*, kanamycin or neomycin) used for selection of plasmids in *Escherichia coli*. The promoter for this gene is only active in bacterial hosts.
2. *lacZ*: A partial *E. coli lacI* coding sequence, the promoter Plac, and a partial coding sequence for beta-d-galactosidase or lacZ protein from pUC119 (Yanisch-Perron *et al.*, 1985).
3. P-MAS: The 0.42 kb TR 2' mannopine synthase promoter region (Velten *et al.* 1984).
4. GUS: the 1.81 kb coding region of the *E. coli* beta-glucuronidase gene (Jefferson *et al.*, 1986). The expression of the gene in plants is used as a scoreable marker for transformation.
5. 7s 3': The 0.43 kn 3' nontranslated region of the soybean 7S seed storage protein alpha subunit (Schuler *et al.*, 1982).
6. FMV 35S: The 0.57 kb figwort mosaic virus 35S promoter (Gowda *et al.*, 1989).

Potential pathogenicity of the donor organism

Only a single new protein, EPSPS, was introduced into soybean variety A5403. The gene encoding this protein was isolated from a naturally occurring soil bacterium, *Agrobacterium* sp. strain CP4. This donor bacterium is not a food source but is related to microbes commonly present in the soil and in the rhizosphere of plants. All plant, microbial, and fungal food sources contain EPSPS proteins, therefore, this enzyme and its activity are not novel to the food supply. *Agrobacterium* strains have also been reported in a number of human clinical specimens, but it is believed that these clinical *Agrobacterium* isolates occur either as incidental inhabitants in the patient or as contaminants introduced during sample manipulation (Kersters and De Ley, 1984).

Characteristics of the donor species, *Agrobacterium*, do not warrant analytical or toxicological tests since only the specific, sequenced gene encoding EPSPS was transferred to soybean. Further detailed information concerning the pathogenicity of other donor organisms is not considered relevant to the risk assessment of GTS 40-3-2 since it was established that only the CP4 EPSPS gene was transferred to the soybean host.

References

Barry, G., G. Kishore, S. Padgette, M. Taylor, K. Kolacz, M. Weldon, D. Re, D. Eichholtz, K. Fincher, & L. Hallas. (1992). Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. In: Biosynthesis and Molecular Regulation of Amino Acids in Plants. B. K. Singh, H. E. Flores, and J. C. Shannon (eds.). American Society of Plant Physiologists pp.139-145.

Beck, E., Ludwig, G., Auerswald, E.A., Reiss, B. & Schaller, H. (1982). Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19, 327-336.

Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., Bittner, M. L.,

Brand, L.A., Fink, C. L., Fry, J. S., Galluppi, G. R, Goldberg, S. B., Hoffman, N. L. & Woo, S. C. (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci.* USA 80, 4803-4807.

Gowda, S., Wu, F.C. & Shepard, R.J. (1989). Identification of promoter sequences for the major RNA transcripts of figwort mosaic and peanut chlorotic streak viruses (caulimovirus group). *J. Cell. Biochem.* 13D (supplement), 301.

Jefferson, R.A, Burgess, S.M. & Hirsch, D. (1986). β-glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci.* USA 83, 8447-8451.

Kay, R., Chan, A., Daly, M. & McPherson, J. (1987). Duplication of the CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* 236, 1299-1302.

Kersters, K., & De Ley, J. (1984). In: Bergey's Journal of Systematic Bacteriology, Vol. l. Kreig, N. R. & Holt, J. G. (eds.). Williams and Wilkins, Baltimore. pp. 244-248.

Odell, J. T., Nagy, F. & Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313, 810-812.

Padgette, S. R., Barry, G.F., Re, D.B., Weldon, M., Eichholtz, D.A., Kolacz, K.H. & Kishore, G.M. (1993). Purification, cloning, and characterization of a highly glyphosate tolerant EPSP synthase from *Agrobacterium* sp. strain CP4. Monsanto Technical Report MSL-12738, St. Louis.

Schuler, M. A., Schmitt, E.S. & Beachy, R.N. (1982). Closely related families of genes code for alpha and alpha prime subunits of the soybean 7S storage protein complex. *Nucl. Acids Res.* 10, 8225-8244.

Shah D., Horsch, R., Klee, H., Kishore, G., Winter, J., Turner, N., Hironaka, C., Sanders, P., Gasser, C., Aykent, S., Siegel, N., Rogers, S. & Fraley, R. (1986). Engineering herbicide tolerance in transgenic plants. *Science* 233, 478-481.

Velten, J., Velten, L., Hain, R. & Schell, J. (1984). Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J.* 3, 2723-2730.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved Ml3 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103-119.

Description of the genetic modification

Description of the transformation method

Plasmid DNA was introduced into the genome of *G. max* cv. A5403 by the particle acceleration method (particle gun) as described in McCabe *et al.* (1988) and Christou *et al.* (1988). DNA was precipitated onto microscopic gold particles using a calcium phosphate solution, and dried down under a stream of nitrogen. The coated particles were resuspended in ethanol and spread onto a mylar carrier sheet. The mylar sheet was accelerated by the force of vaporization as 10-15 kilovolts were discharged across a water drop. The mylar hit a stainless steel retaining screen which stopped the flight of the sheet but allowed the continued flight of the DNA coated particles. The particles penetrated the target plant cells where the DNA was deposited and incorporated into the cell chromosome.

The transformed cells were incubated on a plant tissue culture medium containing cytokinin and auxin to induce multiple shoot formation. The DNA utilized included a marker gene encoding the beta-glucuronidase (GUS) protein (Jefferson *et al.* 1986). The expression of the GUS protein was used as evidence of transformation as detected by a staining method in which the GUS enzyme converted the substrate 5-bromo-4-chloro-3-indolyl beta-d-glucuronide into a blue precipitate. The vast majority of the shoots which were regenerated from the shoot tip cells did not contain any added genes, therefore GUS screening was necessary to identify the genetically modified tissue. The positive shoots were grown to maturity, and the resulting progeny were screened for glyphosate tolerance (by herbicide spray test) and gene expression.

Plasmid PV-GMGT04

Plasmid PV-GMGT04, used to generate line 40-3-2, contained three genes driven by plant promoters: two CP4 EPSPS genes and a gene encoding beta-glucuronidase (GUS) from *E. coli.* PV-GMGT04 is a pUC-Kan vector derived of the high copy *E. coli* plasmid pUC119 (Vieira & Messing 1987) and was constructed by fusing the 1.3 kb *Fsp*I-*Dra*I pUC119 fragment containing the origin of replication to the 1.3 kb *Sma*I-*Hind*III Klenow-filled fragment from pKC7 (Rao & Rogers 1979), which contains the *nptII* gene. The *nptII* gene is driven by a bacterial promoter, preventing its expression in plant cells.

Table 2. Summary of genetic elements in PV-GMGT04

Genetic element	*Size Kb*	*Function*
P-E35S	0.61	The cauliflower mosaic virus (CaMV 35S) promoter with the duplicated enhancer region.
CTP4	0.22	The N-terminal 0.22 kb chloroplast transit peptide sequence from the *Petunia hybrida* EPSPS gene.
CP4 EPSPS	1.36	The C-terminal 1.36 kb 5-enolpyruvylshikimate-3-phosphate synthase gene (CP4 EPSPS) from an *Agrobacterium* species.
NOS 3'	0.26	The 0.26 kb 3' nontranslated region of the nopaline synthase gene.
KAN	1.32	The Tn5 neomycin phosphotransferase type II gene (*nptII*) from the plasmid pKC7. The *nptII* confers kanamycin resistance.
ori-pUC	0.65	The origin of replication from the high copy *E. coli* plasmid pUC119.
LAC	0.24	A partial *E. coli* lacI coding sequence, the promoter Plac, and a partial coding sequence with beta-d-galactosidase or lacZ protein from pUC119.
P-MAS	0.42	The 0.42 kb TR 2' mannopine synthase promoter region.
GUS	1.81	The 1.81 kb coding region of the *E. coli* beta-glucoronidase gene. The expression of the gene in plants is used as a scoreable marker for transformation.
7S 3'	0.43	The 0.43 kb 3' nontranslated region of the soybean 7S seed storage protein alpha subunit.
CMoVb	0.57	The 0.57kb figwort mosaic virus 35S promoter.

Prior to their combination in a single vector, the CP4 EPSPS and GUS genes were assembled with promoters and 3' sequences in the following steps: the CTP4:CP4 EPSPS fusion was combined with the CMoVb promoter and NOS 3' terminator (Fraley *et al.* 1983) and the GUS gene (already fused to the MAS promoter and 7S 3') in vector pMON13615. The CTP4:CP4 EPSPS fusion was then combined with the E35S (CMoVa) promoter and NOS 3' terminator in plasmid pMON13620 where the entire fusion product was flanked by *Hind*III recognition sequences to facilitate further subcloning. These three elements were then combined in pUC plasmid pMON13639 by subcloning the E35S/CTP4:CP4 EPSPS/NOS 3' fusion product from pMON13620. The *Not*I fragment of pMON13639, which has the CP4 EPSPS and GUS elements, was moved into pMON10081, a derivative of pUC119 which contains the origin of replication (*ori*-pUC) and the *nptII* gene. The resulting vector was PV-GMGT04 (Fig. 1).

Extensive restriction analysis of the plasmid PV-GMGT04 and its progenitor plasmids demonstrated that all of the genetic elements and restriction fragments were correctly assembled and produced the correctly sized DNA fragments (Eichholtz *et al.* 1993). A summary of the genetic elements used to assemble plasmid PV-GMGT04 is presented in Table 2. The cloning performed to construct plasmid PV-GMGT04 was done in nonpathogenic *E. coli* strains LE392, JM101 and MM294.

CP4 EPSPS is a 47.6 kD protein consisting of a single polypeptide of 455 amino acids (Padgette *et al.* 1993). The deduced amino acid sequence is shown in

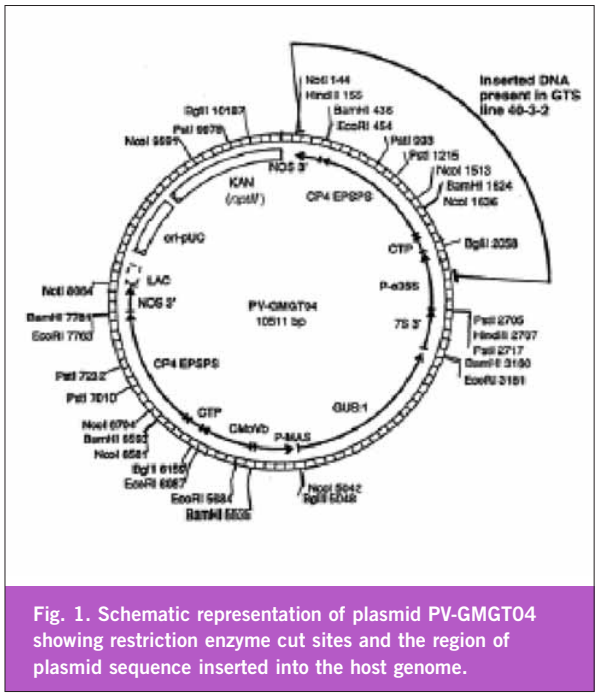


Fig. 1. Schematic representation of plasmid PV-GMGT04 showing restriction enzyme cut sites and the region of plasmid sequence inserted into the host genome.

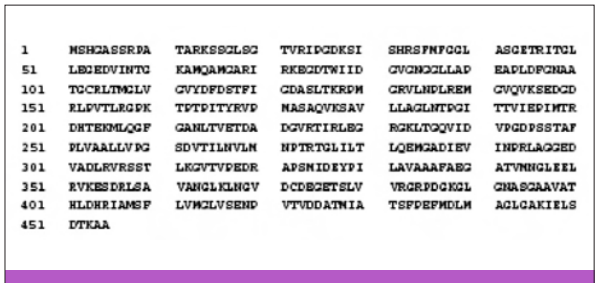


Fig. 2. Deduced amino acid sequence of the *Agrobacterium* sp. Strain CP4 EPSPS gene from pMON17081.

Fig. 2. The identification of codons in the gene encoding four peptide sequences obtained directly from the purified enzymatically-active CP4 EPSPS conclusively demonstrated that the gene cloned was the EPSPS gene from *Agrobacterium* sp. strain CP4.

References

Arencibia, A.D., Carmona, E.R., Tellez, P., Chan, M.T. Yu, S.M., Trujillo, L.E. & Oramas, P. (1998). An efficient protocol for sugarcane (Saccharum spp. L.) transformation mediated by *Agrobacterium tumefaciens. Transgenic Research* **7**, 1-10.

Birch, R.G. (1997). Plant transformation: Problems and strategies for practical application. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 297-326.

Cheng, M, Fry, J.E., Pang, S.Z., Zhou, H.P., Hironaka, C.M., Duncan, D.R., Conner, W. & Wan, Y.C. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens. Plant Physiology* **115**, 971-980.

Cheng, XY, Sardana, R., Kaplan, H. & Altosaar, I. (1998). Agrobacterium-transformed rice expressing synthetic cry1Ab and cry1Ac genes are highly toxic to striped stem borer and yellow stem borer. *Proc. of the Natl. Acad. of Sci.* **95**, 2767-2772.

Christou, P., McCabe, D.E. & Swain, W.F. (1988). Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiology* **87**, 671-674.

Cooley, J., Ford, T. & Christou, P. (1995). Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. *Theor. Appl. Genet.* **90**, 97-104.

Eichholtz, D.A., Barry, G.F., Taylor, M.L. & Padgette, S.R. (1993). Construction of DNA vectors for the production of glyphosate-tolerant soybeans. Monsanto Technical Report MSL-12905, St. Louis, MO.

Enríquez-Obregón, G.A., Vázquez-padrón, R.I., Prieto-sansonov, D.L., de la Riva, G.A. & Selman-Housein,G. (1998). Herbicide resistant sugarcane (*Saccharum officinarum L.*) plants by *Agrobacterium*-mediated transformation. *Planta* **206**, 20-27.

Enríquez-Obregón, G.A., Vázquez-Padrón, R.I., Prieto-Samsónov, D.L., Pérez, M. & Selman-Housein, G. (1997). Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidants compounds. *Biotecnología Aplicada* **14**, 169-174.

Fagard, M. & Vaucheret, H. (2000). (Trans) gene silencing in plants: How many mechanisms? *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* **51**, 167-194.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. & Woo, S.C. (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci.* **80**, 4803-4807.

Gelvin, S.B. (1998). The introduction and expression of transgenes in plants. Current Opinion in Biotechnology 9, 227-232

Hiei, Y., Ohta, S., Komari, T. & Kumashiro, T. (1994). Efficient transformation of rice (*Oriza sativa*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* **6**, 271-282.

Hooykaas, P.J.J. & Shilperoort, R.A. (1992). *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology* **19**, 15-38.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. & Kumashiro, T. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* **4**, 745-750.

Jefferson, R.A., Burgess, S.M. & Hirsch, D. (1986). Beta-glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* **83**, 8447-8451.

Kononov, M.E., Bassuner, B. & Gelvin, S.B. (1997). Integration of T-DNA binary vector "backbone" sequences into the tobacco genome: evidence for multiple complex patterns of integration. *The Plant Journal* **11**, 945-957.

Matzke, A.J.M. & Matzke, M.A. (1998). Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biology* **1**, 142-148.

May, G.D., Afza, R., Mason, H.S., Wiecko, A., Novak, F.J. & Arntzen, C.J. (1995). Generations of transgenic Banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Biotechnology* **13**, 486-492.

McCabe, D.E., Swain, W.F., Martinell, B.J. & Christou, P. (1988). Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology* **6**, 923-926.

Padgette, S.R., Re, D.B., Gasser, C.S., Eichholtz, D.A., Frazier, R.B., Hironaka, C.M., Levine, E.B., Shah, D.M., Fraley, R.T. & Kishore, G.M. (1993). Purification, cloning, and characterization of a highly glyphosate-tolerant EPSP synthase from

Agrobacterium sp. strain CP4. Monsanto Technical Report MSL-12738, St. Louis, MO.

Powlowski, W.P. & Somers, D.A. (1996). Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Molecular Biotechnology* **6**, 17-30.

Powlowski, W.P. & Somers, D.A. (1998). Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. *Proc. Natl. Acad. Sci.* **95**, 12106-12110.

Ramanathan, V. & Veluthambi, K. (1995). Transfer of non-T-DNA portions of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of TL-DNA. *Plant Molecular Biology* **28**, 1149-1154.

Rao, R.N. & Rogers, S.G. (1979). Plasmid PKC7: A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. *Gene* **7**, 79.

Tempe, J. & Schell, J. *In:* Translation of Natural and Synthetic Polynucleotides, A.B. Legocki, Ed. (Poznan University of Agriculture, Poznan, Poland, 1977) p.416.

Vieira, J. & Messing, J. (1987). Production of single-stranded plasmid DNA. *Methods in Enzymology* **153**, 3-11.

Wenck, A., Czako, M., Kanevski, I. & Marton, L. (1997). Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. *Plant Molecular Biology* **34**, 913-922.

Characterization of the genetic modification

Characterization of the primary insert

In order to determine the number of insertion sites of PV-GMGT04 DNA in line 40-3-2, genomic DNA isolated from 40-3-2 and control line A5403 (Dellaporta *et al.* 1983) was digested with *Spe*I and subjected to Southern blot analysis (Southern 1975). The blot was probed with 32P-labelled PV-GMGT04, which does not contain a restriction site for *Spe*I. Line 40-3-2 DNA produced a single band of high molecular weight DNA that was absent from the control lane (Fig. 3, lanes 2 and 3). These results suggest that PV-GMGT04 DNA is present at a single site in 40-3-2 genomic DNA. Three additional bands of lighter intensity, present in both the 40-3-2 and A5403 lanes, represent naturally-occurring cross-hybridizing sequences in A5403 soybean.

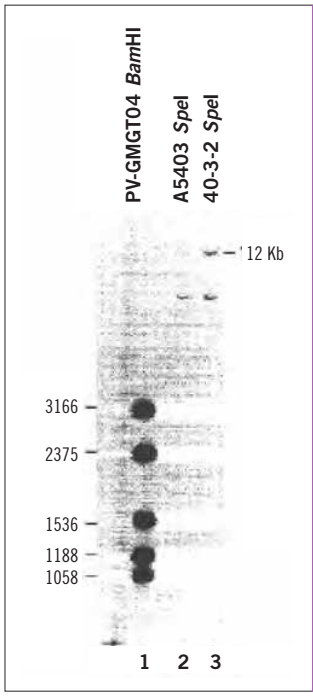


Fig. 3. Southern blot analysis of PV-GMGT04 plasmid DNA digested with *Bam*HI (lane 1), and soybean genomic control A5403 DNA (lane 2) and GTS 40-3-2 genomic DNA (lane 3) digested with *Spe*I. Each lane represents approximately 100 pg of plasmid DNA or approximately 5 ug of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ^{32}P labelled PV-GMGT04 plasmid DNA and subjected to autoradiography.

The number of insertion sites and the approximate size of inserts were also investigated by Southern blot analyses using three restriction enzymes that cut within the plasmid PV-GMGT04. Genomic DNA from GTS 40-3-2 and A5403 was digested with *Bam*HI, *Hind*III, and *Eco*RI, and the separated fragments probed with 32P-labelled PV-GMGT04.

Table 3 lists the predicted sizes of fragments of *Bam*HI, *Hind*III and *Eco*RI digested PV-GMGT04, as well as the sizes of the bands observed for 40-3-2 (Fig. 4, lanes 3, 5 and 7). For *Bam*HI-digested PV-GMGT04 (Fig. 4, lane 1) the observed 1.2 kb fragment corresponded to an anticipated 1.2 kb fragment of PV-GMGT04 (Fig. 4, lane3). The two additional hybridizing bands (Fig. 4, lane 3), which do not match in size to any band in the *Bam*HI PV-GMGT04 digest, are border fragments which contain part of the plasmid DNA attached to plant genomic DNA. *Hind*III cuts twice within PV-GMGT04 but only one hybridizing band was detected for 40-3-2 (Fig. 4, lane 5), indicating that at least one or both *Hind*III sites were absent from the insert. As shown in Fig. 1, an *Eco*RI site is present in the 1.2 kb CP4 EPSPS *Bam*HI fragment of PV-GMGT04. Two bands were observed for *Eco*RI digested 40-3-2 DNA (Fig. 4, lane 7), indicating that *Eco*RI cuts once within the CP4 EPSPS gene of the insert to generate two border fragments. The presence of no more than two border fragments for *Bam*HI, *Hind*III and *Eco*RI digested 40-3-2 DNA confirms the presence of a single insertion site. The total size of the hybridizing bands was less than 6 kb in the three

digestions, indicating that a PV-GMGT04 fragment of less than 6 kb was integrated into the plant genome.

A combination of PCR and Southern blot analyses was used to characterize the single insert present in line 40-3-2.

ori-pUC

To analyze for the presence of the pUC origin of replication (*ori*-pUC), oligonucleotides corresponding to the 5' and 3' sequences of ori-pUC were used in a polymerase chain reaction (PCR) analysis (Mullis & Faloona l987; McPherson *et al.* 1991) of genomic DNA from 40-3-2, 61-137 and A5403. 61-137 is an experimental glyphosate tolerant soybean line, transformed with the plasmid PV-GMGT04 and known to contain sequences corresponding to the ori-pUC region. As shown in Fig. 5, DNA from line 61-137 and PV-GMGT04 produced bands of the expected size of 671 bp (lanes 4 and 5). No bands of this size were observed for either 40-3-2 or the control A5403 (lanes 2 and 3). These results established that an intact *ori*-pUC element was not present in line 40-3-2.

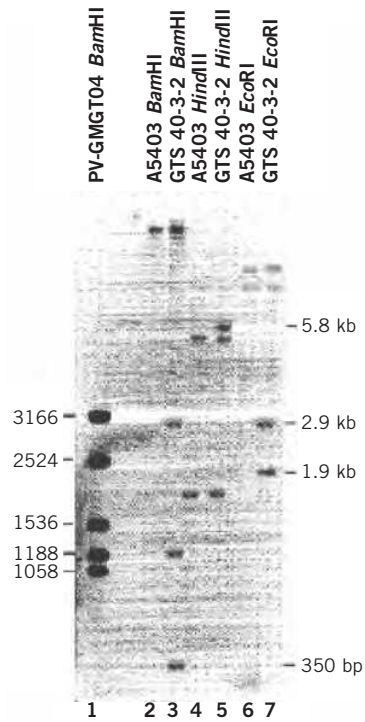


Fig. 4. Southern blot analysis of PV-GMGT04 DNA digested with *Bam*HI (lane 1), soybean A5403 control DNA digested with *Bam*HI (lane 2), *Hind*III (lane 4) and *Eco*RI (lane 6), and 40-3-2 DNA digested with *Bam*HI (lane 3), *Hind*III (lane 5) and *Eco*RI (lane 7). Each lane represents approximately 100 pg of plasmid DNA or approximately 5 ug of genomic DNA. DNA was subjected to electrophoresis through a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with 32P labelled PV-GMGT04 plasmid DNA and subjected to autoradiography.

Table 3. Restriction analysis of line 40-3-2 and plasmid PV-GMGT04

***Restriction fragments size (bp)*[1]**

Bam*HI*		Hind*III*		Eco*RI*	
Plasmid	*40-3-2*	*Plasmid*	*40-3-2*	*Plasmid*	*40-3-2*
3166		7959		3202	
	2900		5800		2900
2375		2552		2727	
1536				2503	
1188	1200				1900
1058				1646	
	350			403	

1. The values for the plasmid PV-GMGT04 are based on calculated sizes (Fig. 1). The values for 40-3-2 are estimated from gel migration relative to molecular weight markers (Fig. 4). Bands present in both the experimental and control lanes are not listed.

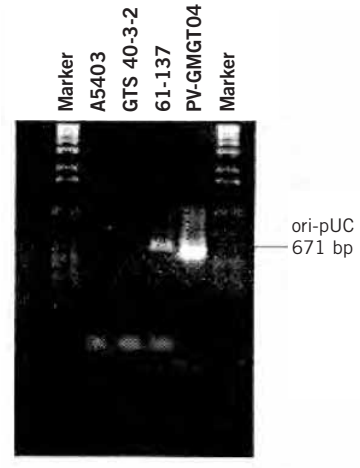


Fig. 5. PCR analysis of line 40-3-2 genomic DNA for ori-pUC. Genomic DNA from control line A5403 and event 40-3-2 were analyzed using PCR to determine the presence or absence of the pUC origin of replication. The positive DNA controls were PV-GMGT04 plasmid DNA and 61-137, a soybean line containing ori-pUC. A 5' and a 3' oligonucleotide were made identical to the 5- and 3'ends of ori-pUC. Reactions were done in 100 ul total volume containing 100 pg of each oligo, 1 ug template, dNTPs at 200 uM, 10 units of Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 55°C annealing for 1.5 min, and a 72°C extension for 3 min. The cycle was repeated 24 times. Products were separated on a 1.25% agarose gel and visualized by ethidium bromide staining. The lower bands at the bottom of the gel are unused oligos.

nptII

PCR analysis was also used to test for the presence of the *nptII* gene in line 40-3-2. Four oligonucleotides were used: 5' and 3' oligonucleotides corresponding to the ends of the *nptII* gene, and 5' and 3' oligonucleotides internal to the gene. Genomic DNA from 40-3-2, 61-137 and A5403, and PV-GMGT04 plasmid DNA was used as template. The oligonucleotides were used in four combinations: 5' end and 3' end; 5' end and 3' internal; 3' end and 5' internal; and both internal primers. As shown in Fig. 6, PV-GMGT04 (lanes 5 and 11) and 61-137 (lanes 6 and 12) produced the correct size PCR products. Lines 40-3-2 (lanes 3 and 9) and A5403 (lanes 2 and 8) showed none of the predicted *nptII* PCR products. These results established that an intact *nptII* gene was not present in line 40-3-2.

CP4 EPSPS

Genomic DNA from A5403 and 40-3-2 was digested with *Hind*III, or *Bg*lII/*Eco*RI. The blot was hybridized with a 32P-labelled probe specific to the CP4 EPSPS coding region. A 5.8 kb band of *Hind*III digested 40-3-2 DNA hybridized with the CP4 EPSPS gene (Fig. 7, Panel A, lane 5), indicating that the CP4 EPSPS gene (or gene fragment) was present in line 40-3-2. This 5.8 kb band was also evident in Fig. 4 (lane 5). The CP4 EPSPS probe

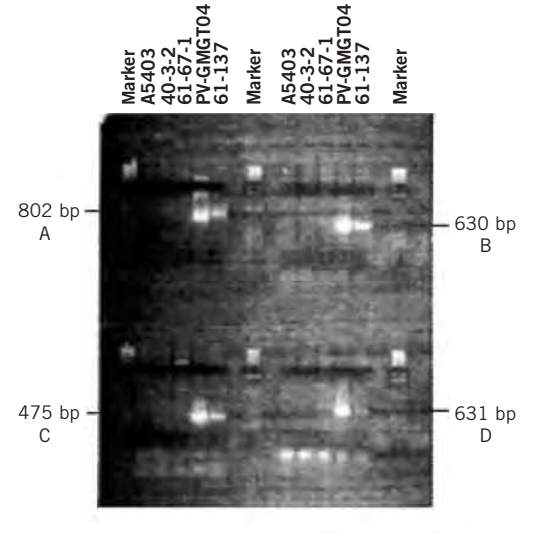


Fig. 6. PCR analysis of line 40-3-2 genomic DNA for nptII. Soybean genomic DNA from the GTS 40-3-2 was analyzed using PCR to determine the presence or absence of the *npt*II gene. The negative controls were A5403 and 61-67-1, an experimental GTS line negative for nptII. Two positive controls were used: PV-GMGT04 plasmid DNA and 61-137, a GTS line positive for nptII. Four oligonucleotides were used in this analysis: a 5' and a 3' oligo were made identical to the ends of the gene, and a 5' and a 3' oligo were made identical to internal sequences of the gene: nptII 5' (nt 10159 to 10140), nptII 5' internal (nt 10005 to 9988), nptII 3' end (nt 9357 to 9370), and nptII 3' internal (nt 9511 to 9529). The predicted product sizes are: A= 5' end + 3' end, 802 bp; B = 5' end + 3' internal, 630bp; C = 5' internal + 3' internal, 475 bp; and D = 5' internal + 3' end 631 bp. Reactions were done in 100ul total volume, containing 100 pg of each indicated oligo, 1 ug template, dNTPs at 200 uM, 10 units Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 63°C annealing for 1.5 min, and a 72°C extension for 6 min. The cycle was repeated 24 times. Products were separated on a 1.25% agarose gel and visualized by ethidium bromide staining. The lower bands at the bottom of each gel are unused oligos.

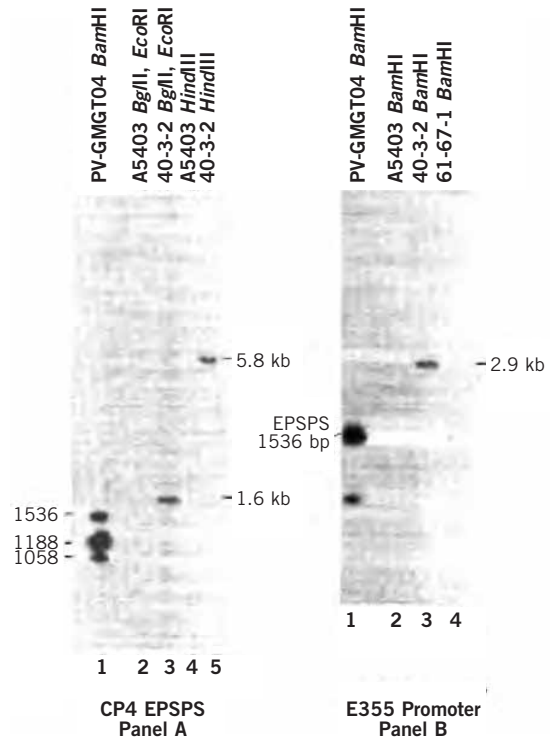


Fig. 7. Southern blot analysis with CP4 EPSPS and E35S probes. PV-GMGT04 plasmid DNA was digested with *Bam*HI (lane 1 in both panels). Genomic DNA from A5403 control was digested with *Bg*lII/*Eco*RI (panel A, lane 2), *Hind*III (panel A, lane 4) and *Bam*HI (panel B, lane 2). GTS line 40-3-2 DNA was digested with BglII/EcoRI (panel A, lane 3), *Hind*III (panel A, lane 5), and *Bam*HI (panel B, lane 3). GTS 61-67-1, a negative control for E35S was digested with *Bam*HI (panel B, lane 4). Each lane represents approximately 100 pg of plasmid DNA or approximately 5 ug of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ^{32}P-labelled coding region of CP4 EPSPS (panel A), or E35S promoter (panel B), and then subjected to autoradiography. The smaller mark in lane 1 of panel B is a dot on the blot and not an additional band.

was predicted to hybridize with a 2552 bp band of *Hind*III-digested PV-GMGT04 DNA (Fig. 4.1). No fragment of this size was detected for 40-3-2, indicating that at least one of the PV-GMGT04 *Hind*III sites was not transferred to line 40-3-2. A band of 1.6 kb *Bg*lII/+*Eco*RI-digested 40-3-2 DNA (Fig. 7, Panel A, lane 3) hybridized with the CP4 EPSPS probe, indicating that an intact CP4 EPSPS gene was present in 40-3-2.

E35S promoter

A Southern blot was performed using A5403 and 40-3-2 DNA digested with *Bam*HI, and probed with 32P-labelled E35S promoter DNA. The E35S element, or a portion of it, was present in line 40-3-2 (Fig. 7, Panel B, lane 3); a single band of 2.9 kb was detected for 40-3-2, corresponding to the border fragment detected in Fig. 4 (lane 3) and discussed above. Since E35S is located on a 1536 bp *Bam*HI fragment of PV-GMGT04 (Fig. 1), and no fragment of this size was detected for 40-3-2, it is clear that the *Bam*HI site at nucleotide (nt) 3160 (Fig.1) was not present in line 40-3-2.

NOS 3'

A Southern blot was performed using A5403 and 40-3-2 DNA digested with *Hind*III, and probed with 32P-labelled NOS 3' terminator DNA. At least a portion of the NOS 3' element is present in 40-3-2 (Fig. 8, lane 10) as a single band of 5.8 kb was detected for line 40-3-2, corresponding to the border fragment detected in Fig. 5.2 (lane 5) and discussed above. A5403 and 40-3-2 DNA was subsequently digested with *Eco*RI/*Bg*lII and *Eco*RI/*Hind*III. A 0.8 kb fragment of *Eco*RI/*Hind*III digested 40-3-2 DNA hybridized with the NOS 3' probe (lane 5) where the map predicted size is 0.3 kb. A 1.2 kb fragment of *Eco*RI/*Bg*lII digested 40-3-2 DNA hybridized to the NOS 3' (lane 3) probe where the predicted size is 0.8 kb. These results indicate that the *Hind*III site at nt 155 and the *Bg*lII site at nt 10187 were not present in the insert of 40-3-2.

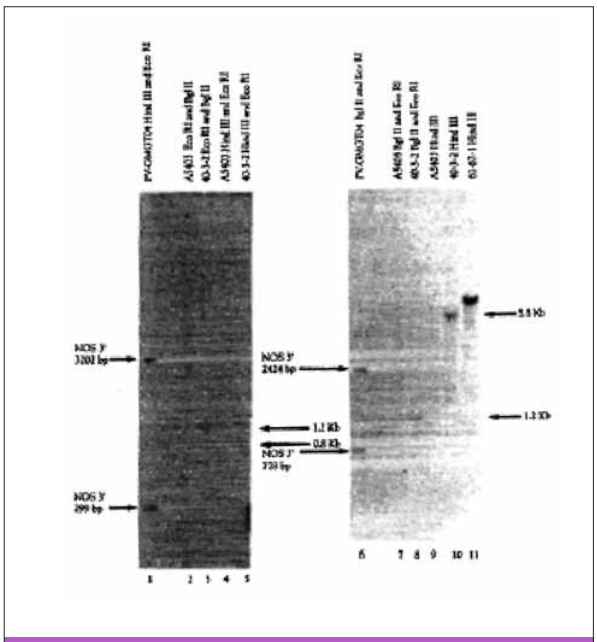


Fig. 8. Southern blot analysis with NOS 3' probe. PG-GMGT04 plasmid DNA was digested with *Hind*III/EcoRI (lane 1) and *Bg*lII/*Eco*RI (lane 6). Genomic DNA from A5403 control was digested with *Eco*RI/*Bg*lII (lanes 2 and 7), with *Hind*III/EcoRI (lane 4), and with *Hind*III (lane 9). GTS line 40-3-2 was digested with *Bg*lII/*Eco*RI (lanes 3 and 8), with *Hind*III and EcoRI (lane 5) and with *Hind*III (lane 10). GTS line 61-67-1, a positive control for NOS 3' was digested with *Hind*III (lane 11). Each lane represents approximately 100 pg of plasmid DNA or approximately 5 ug of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. Both panels were probed with 32P labelled NOS 3' and then subjected to autoradiography.

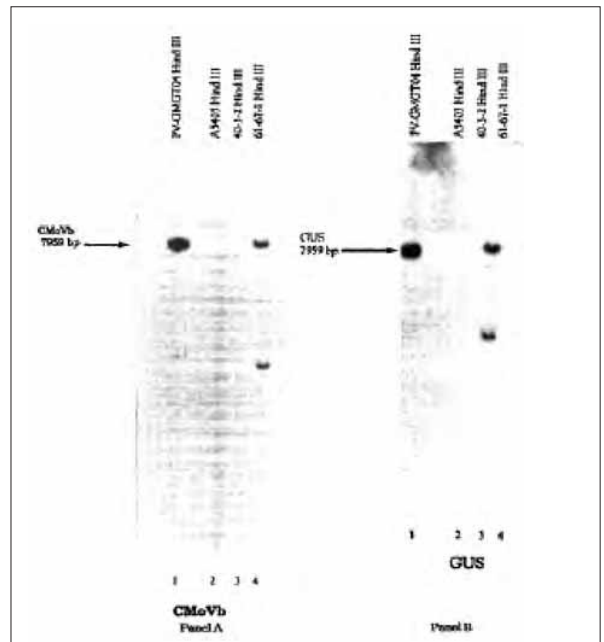


Fig. 9. Southern blot analysis with CMoVb and GUS probes. PV-GMGT04 plasmid DNA was digested with *Hind*III (panels A and B, lanes 1). Soybean A5403 control DNA was digested with *Hind*III (panels A and B, lanes 2). GTS line 40-3-2 DNA was digested with *Hind*III (panels A and B, lanes 3), and GTS line 61- 67-1 DNA was digested with *Hind*III (panels A and B, lane 4). Each lane represents approximately 100 pg plasmid DNA or approximately 5 pg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with 32P labelled CMoVb promoter (panel A) or the coding region of GUS (panel B) and then subjected to autoradiography.

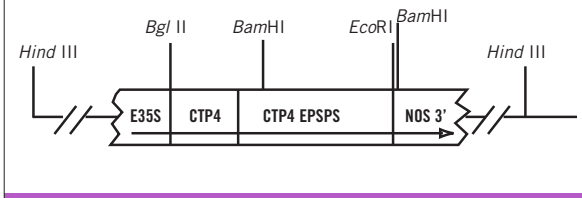


Fig. 10. Predicted DNA insert in soybean event 40-3-2 located on a 5.8 kb *Hind*III restriction fragment.

CMoVb promoter

A Southern blot was performed using A5403 and 40-3-2 DNA digested with *Hind*III, and probed with 32P-labelled CMoVb promoter. As shown in Fig. 9, no band was detected for line 40-3-2 (Panel A, lane 3), indicating that the CMoVb promoter DNA is not present in line 40-3-2. GTS line 61-67-1, which contains the CMoVb promoter, provided a positive control (Panel A, lane 4).

GUS

A Southern blot was performed using A5403 and 40-3-2 DNA digested with *Hind*III, and probed with ^{32}P-labelled GUS coding region. As shown in Fig. 9, no band was detected for 40-3-2 (Panel B, lane 3), indicating that GUS is not present in this line. GTS line 61-67-1, which contains the GUS gene, provided a positive control (Panel B, lane 4).

Characterization of the secondary insert

Additional characterization of GTS 40-3-2 was undertaken using a Southern blot method with higher sensitivity than that used in the initial characterizations (Re *et al.* 1993; Kolacz & Padgette 1994; Padgette *et al.* 1996). DNA from event 40-3-2 and the R3 progeny generation (Resnick BC1F2) used to develop commercial varieties was digested with the restriction enzyme *Hind*III and subjected to Southern blot hybridization analysis using a full length CP4 EPSPS coding sequence probe. A5403 control DNA and A5403 control DNA spiked with plasmid PV-GMGT04 DNA were also digested with *Hind*III and used as controls. The results are shown in Fig. 11. A5403 control DNA (lane 2) showed no hybridization bands, as expected, while A5403 control DNA spiked with plasmid PV-GMGT04 DNA (lane 3) produced two bands at ~2.5 kb and ~8.0 kb as predicted from the plasmid map in Fig. 1. Resnick BC1F2 DNA (lane 4) and event 40-3-2 DNA (lane 5) produced the expected size band at approximately 5.8 kb, which represents the primary, functional insert, as well as a band at approximately 900 bp. There is a slight difference in the migration of the ~900 bp band between the two samples due to variations in DNA quality.

To more clearly define the region of CP4 EPSPS present on the ~900 base pair *Hind*III restriction fragment, genomic DNA extracted from both 40-3-2 and Resnick BC1F2 material was analyzed by Southern blot hybridization with sequential portions of the CP4 EPSPS coding sequence and the NOS 3' transcriptional termination element (see diagram at bottom of Fig. 12). A5403 control DNA, A5403 control DNA spiked with plasmid PV-GMGT04 DNA, Resnick BC1F2 DNA, and 40-3-2 DNA were digested with *Hind*III and included on all Southern blots. Southern blot analyses on the Resnick BC1F2 and 40-3-2 DNA samples performed using the NOS 3' probe and three CP4 EPSPS probes (Probe-1, Probe-2, and Probe-4) generated only the expected band at ~5.8 kb representing the primary, functional insert in

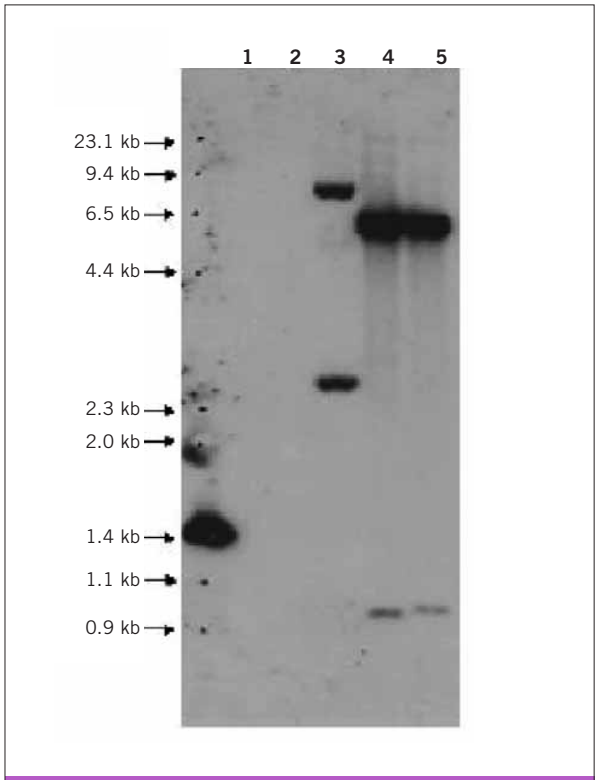


Fig. 11. Southern blot analysis of event 40-3-2. Ten micrograms of genomic DNA extracted from leaf tissue of A5403 control (lane 2), A5403 control spiked with ~15 pg PV-GMGT04 plasmid DNA (lane 3), Resnick BC1F2 (lane 4), and 40-3-2 (lane 5) were digested with *Hind*III. Lane 1 was left blank. The blot was probed with the ^{32}P-labelled full length CP4 EPSPS coding region. The arrow symbol denotes sizes obtained from MW markers on ethidium stained gel.

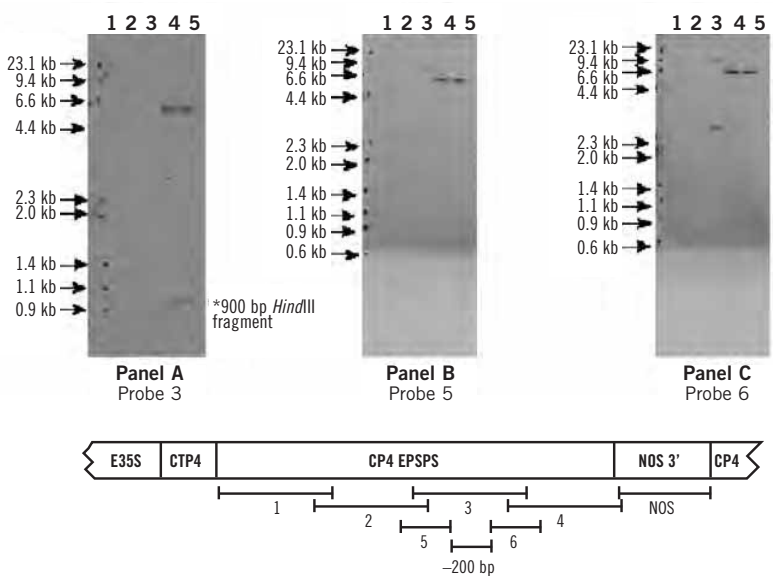


Fig. 12. Southern blot analysis using overlapping CP4 EPSPS probes. Ten micrograms of genomic DNA extracted from leaf tissue of A5403 control (lane 2), A5403 control spiked with ~15 pg PV-GMGT04 plasmid DNA (lane 3), Resnick BC1F2 (lane 4), and 40-3-2 (lane 5) were digested with *Hind*III. Lane 1 is blank in all panels. Panel A was probed with CP4 EPSPS probe-3, panel B with CP4 EPSPS Probe-5, and panel C with CP4 EPSPS probe 6. The blot in panel A is the result of stripping and reprobing of the blot in Fig. 11. The positions of the probes with respect to the CP4 EPSPS coding sequence and NOS are illustrated on the linear map below the panels with the probes used in panels A, B, and C in bold print. The arrow symbol denotes sizes obtained from MW markers on ethidium stained gel.

soybean event 40-3-2 (data not shown). The only Southern blot on which the ~900 bp HindIII restriction fragment was observed in the Resnick BC1F2 DNA and 40-3-2 DNA samples is shown in Fig. 12, Panel A. This blot was probed with CP4 EPSPS Probe-3 (see diagram at bottom of Fig. 12). The blot in Fig. 11 was stripped and reprobed to generate this result, therefore the size of the ~900 bp HindIII restriction fragment is again slightly shifted between the two soybean event 40-3-2 samples. Probes designed to overlap the 5' and 3' ends of CP4 EPSPS Probe-3 did not hybridize to the ~900 bp *Hind*III fragment (Fig. 12, Panels B and C). These results indicate that the NOS 3' transcriptional termination element is not present on the ~900 bp *Hind*III restriction fragment, and that the portion of the CP4 EPSPS coding region contained within the ~900 bp HindIII restriction fragment is less than 200 bp in length.

To further delineate the CP4 EPSPS sequence present on the ~900 bp *Hind*III restriction fragment, a pool of cosmid DNA which contained the ~900 bp HindIII restriction fragment was digested with *Hind*III, separated by agarose gel electrophoresis and transferred to a nylon membrane. The plasmid vector PV-GMGT04 was used as a positive hybridization control and should result in the visualization of two bands at ~8.0 kb and ~2.5 kb based on the plasmid map (Fig. 1). Several identical blots were hybridized separately with oligonucleotide probes 3'-end labelled with digoxigenin-11-dUTP (see diagram at bottom of Fig. 13). Hybridization of the cosmid DNA was not observed with the oligonucleotide probes Oligo-1, Oligo-2, Oligo-3, Oligo-4, Oligo-8 and Oligo-9, although the probes did hybridize to the PV-GMGT04 plasmid positive control, indicating that the conditions employed were conducive for hybridization (data not shown). However, oligonucleotide probes Oligo-5 and Oligo-6 did hybridize to the ~900 bp *Hind*III restriction fragment in the DNA extracted from the cosmid DNA (data not shown). The pool of cosmid clones was further screened to isolate single colonies that contained the ~900 bp *Hind*III restriction fragment. The purified cosmid clone 6A was digested with *Hind*III, separated by agarose gel electrophoresis, and transferred to a nylon membrane. The controls were identical to those used in the experiment on the cosmid pool. Hybridization was observed between the oligonucleotide probes Oligo-5 and Oligo-6 with the ~900 bp *Hind*III restriction fragment as was previously observed with DNA prepared from the pool. However, oligonucleotide probe Oligo-7 located immediately 3' of the Oligo-6 probe did not hybridize to the ~900 bp *Hind*III restriction fragment in the cosmid DNA prepared from clone 6A. Oligonucleotide probe Oligo-4, located immediately 5' of Oligo-5 probe and used on the pool of cosmid DNA, also did not hybridize to the ~900 bp *Hind*III restriction fragment (Fig. 13). The two oligonucleotide probes, Oligo-5 and Oligo-6, which did hybridize to the ~900 bp *Hind*III restriction fragment in the DNA from cosmid clone 6A, are contiguous in the CP4 EPSPS coding region and represents a minimum of 53 bp of the maximum 200 bp region expected to be present from previous probe walking experiments on soybean event

40-3-2 genomic DNA (Fig. 12). In conclusion, the oligonucleotide probe hybridization to the cosmid clones allowed the portion of the CP4 EPSPS sequence present on the ~900 bp *Hind*III restriction fragment to be defined as ~53 bp consisting of sequence which hybridized to the Oligo-5 and Oligo-6 probes (Fig. 13).

Oligo-5 and Oligo-6 (Fig. 13) were used as primers to generate DNA sequence directly from purified cosmid clones 6A and 4B (a second cosmid clone shown to contain a similar insert to 6A) in both the 5' and 3' directions. Multiple primers were then designed to the resulting potential 5' and 3' flanking sequences and paired with Oligo-5 and Oligo-6 primers. PCR products were obtained and subsequenly sequenced. The combination of DNA sequence data revealed that 72 bp of CP4 EPSPS (base pairs 855-926, Fig. 1) are located on a 937 bp *Hind*III restriction fragment. No other sequences derived from plasmid PV-GMGT04 (Fig. 1) used in the transformation of soybean event 40-3-2 were identified on the 937 bp *Hind*III restriction fragment. A schematic of the additional insert is shown in Fig. 14. The observation that only 72 bp of the CP4 EPSPS sequence are present on the 937 bp *Hind*III restriction fragment explains the low hybridization intensity of this band when compared to the ~5.8 kb *Hind*III restriction fragment containing the primary, functional insert when probed with a full-length CP4 EPSPS probe (Fig. 11, lanes 4 and 5). This observation also accounts for why the additional CP4 EPSPS segment was not observed using less sensitive methods used to characterize the primary insert as described.

PCR analyses were performed on DNA extracted from Resnick BC1F2 and event 40-3-2, as well as isolated cosmid clones 4B and 6A, to demonstrate that the 5' and 3' genomic flanking sequences of the 72 bp CP4 EPSPS segment were consistent in all samples. Three different PCR analyses were performed, including one PCR verifying the 5' genomic flanking sequence using Primers A and B, a second PCR verifying the 3' genomic flanking sequence using Primers A' and C, and a third PCR amplifying from the 5' genomic flanking sequence to the 3' genomic flanking sequence using Primers B and C. The positions of all primers as well as the results of all PCR analyses are shown in Fig. 15. The control reactions without template (lanes 7, 13, and 19) and A5403 non-transgenic negative control DNA (lanes 6, 12, and 18) did not generate a PCR product in any of the analyses. The Resnick BC1F2 DNA samples (lanes 2, 8, and 14), the 40-3-2 samples (lanes 3, 9, and 15), cosmid clone 4B (lanes 5, 11, and 17) and cosmid clone 6A (lanes 4, 10, and 16)

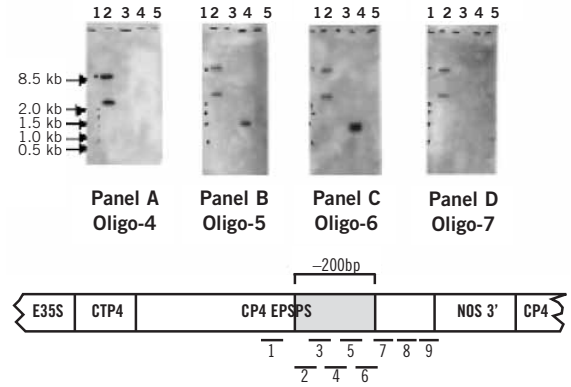


Fig 13. Southern blot analysis with various oligonucleotide probes of cosmid DNA prepared from the isolated cosmid clone 6A. Oligonucleotide probes were 3'-end labelled with digoxigenin-11-dUTP and probed against individual Southern blots of DNA from the purified cosmid clone 6A digested with *Hind*III (lane 4, 4 ng per lane except for panel A where 900 pg of DNA from a pool of cosmid DNA was used). Molecular weight marker DNA was loaded in lane 1 of each panel for size estimation of the bands being observed. The same molecular weight marker was used for each panel. Plasmid PV-GMGT04 digested with the *Hind*III served as a positive control (lane 2, 1 ng per lane). Lanes 3 and 5 of each panel were blank. The positions of the oligonucleotide probes with respect to the CP4 EPSPS coding sequence are illustrated on the linear map below the panels with the probes used in panels A-D in bold print. The shaded ~200 bp region represents the maximum region delineated to be present on the ~900 bp *Hind*III fragment of DNA from 40-3-2 that was observed to hybridize with CP4 EPSPS probe-3. The arrow symbol denotes sized obtained from MW markers on ethidium bromide stained gel.

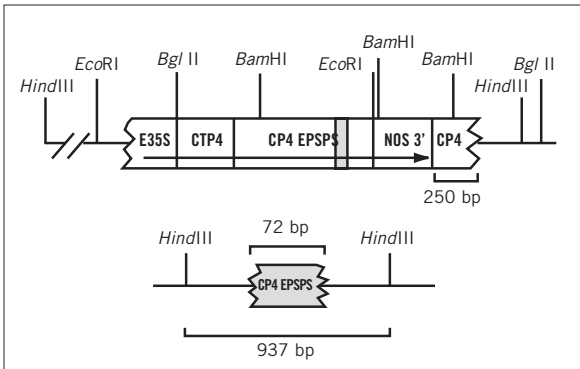


Fig. 14. Predicted DNA inserts in soybean event 40-3-2 based on genome walking, higher sensitivity Southern blot analysis, genomic cloning, nucleotide sequencing and PCR. There is an additional 250 bp segment of the CP4 EPSPS sequence immediately adjacent to the NOS 3' transcriptional termination element on the primary insert and an additional insert located on a 937 bp *Hind*III restriction fragment consisting of 72 bp of the CP4 EPSPS sequence. The shaded region in the CP4 EPSPS sequence in the functional primary insert represents the 72 bp present in the second insert.

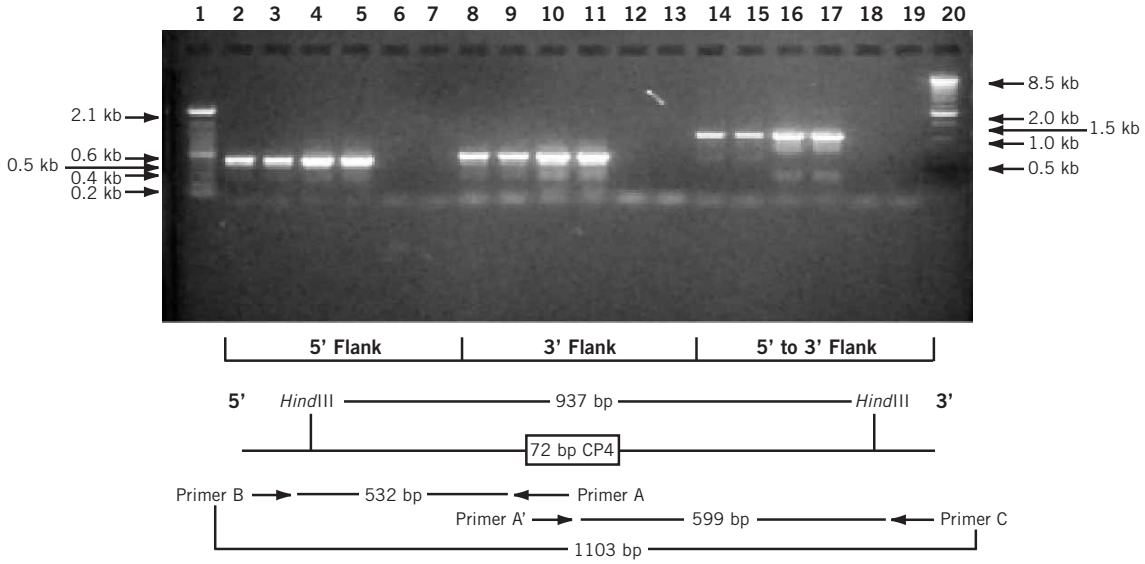


Fig 15. PCR analyses of second insert. PCR analyses were performed using primers A and B to confirm the 5' flanking sequence, primers A' and C to confirm the 3' flanking sequence, and primers B and C to perform PCR from the 5' to 3' flank on DNA extracted from leaf tissue of Resnick BC1F2 (lanes 2, 8, and 14) and 40-3-2 material (lanes 3, 9, and 15), as well as cosmid clones 6A (lanes 4, 10, and 16) and 4B (lanes 5, 11, and 17) DNA. Lanes 1 and 20 contain Gibco BRL 100 bp DNA ladder and 500 bp DNA ladder, respectively. Lanes 6, 12, and 18 contain A5403 non-transgenic DNA PCR reactions and lanes 7, 13, and 19 were no template control PCR reactions. Ten microliters of each PCR reaction was loaded on the gel. The arrow symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

generated the expected specific size PCR products of 532 bp for the 5' flanking sequence, 599 bp for the 3' flanking sequence, and 1103 bp for the 5' to 3' flanking sequence (see diagram at bottom of Fig. 15). The PCR products from similar reactions were subjected to DNA sequencing. The results revealed that the genomic flanking sequence present in cosmid clones 4B and 6A is consistent with the genomic flanking sequence in Resnick BC1F2 material and 40-3-2 material. These results further establish the validity of the cosmid clones used in this analysis and establish that the second insert in event 40-3-2 consists of 72 bp of the CP4 EPSPS element (base pairs 855-926 of PV-GMGT04, Fig. 1) located on a 937 bp *Hind*III restriction fragment with no other sequences from plasmid PV-GMGT04 used in the transformation of the event.

Sequence of the 5' and 3' ends of the primary insert

The PCR-based technique GenomeWalker (CLONTECH, Palo Alto, CA) was used to generate PCR products containing DNA at the 5' and 3' ends of the inserted DNA, as well as the DNA flanking the 5' and 3' ends of the primary insert in soybean event 40-3-2. The PCR products were subjected to DNA sequencing and multiple primers designed to the flanking sequences were paired with insert specific primers located: in the E35S promoter, to validate the sequence at the 5' end of the inserted DNA and the 5' flanking genomic sequence; and in the NOS 3' transcriptional termination element, to validate the DNA sequence at the 3' end of the inserted DNA and the sequence of the 3' flanking genomic DNA. PCR products were obtained and sequenced. The resulting sequences are shown in Fig. 5.14 and Fig. 17. Figure 16 contains the 5' DNA sequence which shows that the first 354 bp of the E35S promoter are missing with the insert beginning at base pair 2347 of PV-GMGT04 (Fig. 1). This deletion removes a duplicated portion of the E35S enhancer region and is not likely to have a significant effect on the functionality of the promoter since the region necessary for transcriptional initiation remains intact (Odell *et al.* 1985). In addition to the 105 bp of E35S which were sequenced, 186 bp of the soybean genomic DNA adjacent to the 5' end of the inserted DNA is shown in Fig. 16. Figure 17 contains the 3' DNA sequence, which demonstrates that the entire NOS 3' transcriptional termination element is present

rather than the partial NOS sequence reported above. Adjacent to the inserted DNA ending at base pair 160 of PV-GMGT04 (Fig.1), a previously unobserved 250 bp portion of the CP4 EPSPS element was identified which consists of base pairs 195-444 in Fig. 17. This sequence corresponds to base pairs 1490-1739 of PV-GMGT04 in Fig. 1. Figure 17 also shows the sequence of 416 bp of flanking soybean genomic DNA. This CP4 EPSPS segment (base pairs 1490-1739 of PV-GMGT04, Fig. 1) does not contain a promoter or 3' transcriptional termination element, therefore transcription and subsequent translation of this region is highly unlikely. A northern blot was conducted which established that no mRNA is detected other than the full-length mRNA. Furthermore, in the highly unlikely event that this region would have been transcribed and translated as a fusion to the full length CP4 EPSPS protein, western blot analysis using antisera to CP4 EPSPS would have resulted in a higher molecular weight protein species being detected. No protein other than the full-length CP4 EPSPS was observed (Rogan *et al.* 1999), strongly suggesting that this DNA sequence is not transcribed or translated as a fusion protein.

Summary

In conclusion, it was determined that GTS 40-3-2 contained two inserted DNA segments, one containing a functional CP4 EPSPS gene construct (partial E35S promoter, chloroplast transit peptide signal sequence, CP4 EPSPS encoding sequence and NOS 3' terminator), and a second smaller insert consisting of 72 bp of CP4 EPSPS sequence. Additionally, sequencing of soybean genomic DNA flanking the functional CP4 EPSPS insert confirmed a deletion in the E35S enhancer region. The region known to be critical for proper transcriptional initiation was not disturbed. Sequencing of the NOS 3' transcriptional termination element and the flanking plant DNA revealed that the NOS sequence is intact. An additional 250 bp segment of the CP4 EPSPS element adjacent to the 3' end of the NOS 3' transcriptional termination element was shown to be present. Since neither a promoter nor a 3' transcriptional termination element is evident within either of the small CP4 EPSPS segments, it is extremely unlikely that these regions would be transcribed. Furthermore, northern blot and western blot data show that only the expected CP4 EPSPS full-length transcript and protein are detected, respectively. These data support the conclusion that neither transcription nor translation of these CP4 EPSPS DNA segments occurs.

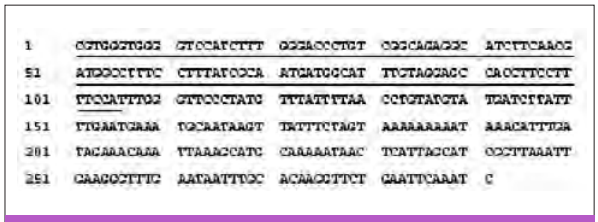


Fig. 16. 5' flanking sequence of the primary insert in soybean event 40-3-2. The underlined base pairs 1-105 (corresponding to bp 2241-2347 of PV-GMGT04, Fig. 1) represent a portion of the E35S promoter. Base pairs 106-291 represent flanking soybean genomic DNA.

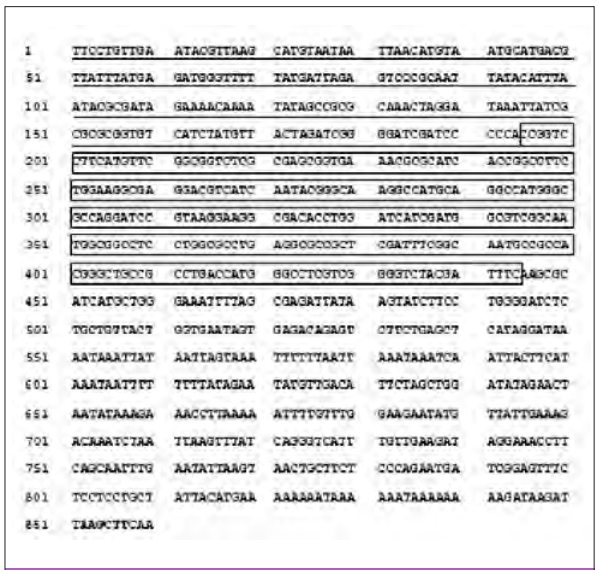


Fig. 17. 3' flanking sequence of he primary insert in soybean event 40-3-2. The underlined base pairs 1-194 (corresponding to base pairs 160-353 of PV-GMGT04, Fig. 4.1) represent the 3' portion of the NOS 3' transcriptional termination element present within the functional insert, along with 16 base pairs of plasmid PV-GMGT04 (italics) immediately adjacent to NOS. The boxed region at base pairs 195-444 (corresponding to base pairs 1490-1739 of PV-GNGT04, Fig. 1) delineates 250 bp of the CP4 EPSPS coding region. Base pairs 445-860 represent flanking soybean genomic DNA with a *Hind*III site indicated in bold letters beginning at base pair 852.

References

Dellaporta, S.L., Wood, J. & Hicks, J.B. (1983). A plant DNA minipreparation: Version II. *Plant Mol. Biol. Reporter* **1**, 19-21.

Kolacz, K.H. & Padgette, S.R. (1994). Molecular characterization of the 5' and 3' ends of the inserted DNA and the stability of the insert in glyphosate-tolerant soybean line 40-3-2. Monsanto Study #93-01-30-43, Monsanto Technical Report MSL-13524, St. Louis, MO.

McPherson *et al.* eds. (1991). PCR: A Practical Approach. Oxford University Press, Oxford, pp. 1-13.

Mullis, K & Faloona, F. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**, 335-350.

Odell, J.T., Nagy, F. & Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313**, 810-812.

Padgette, S.R., Taylor, N.B., Nida, D.L., Bailey, M.R., MacDonald, J., Holden, L.R. & Fuchs, R.L. (1996). The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. *Journal of Nutrition* **126**, 702-716.

Re, D.B., Padgette, S.R., Delannay, X., Kolacz, K.H., Nida, D.L., Peschke, V.M., Derting, C.W., Rogers, S.G., Edwards, J.W., Barry, G.F. & Biest, N.A. (1993). Petition of determination of nonregulated status: soybeans with a Roundup Ready gene. Submitted to USDA, St. Louis.

Rogan, G.J., Dudin, Y.A., Lee, T.C., Magin, K.M., Astwood, J.D., Bhakta, N.S., Leach, J.N., Sanders, P.R. & Fuchs, R.L. (1999). Immunodiagnostic methods for detection of 5-enolpyruvylshikimate-3-phosphate synthase in Roundup Ready soybeans. *Food Control* **10**, 407-414.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Molecular Biology* **98**, 503-517.

Genetic stability of the introduced trait

The stable integration of the CP4 EPSPS gene into the genome of GTS 40-3-2 was demonstrated through a combination of molecular (*e.g.*, Southern blotting, PCR analysis, and protein expression) and phenotypic trait segregation analyses.

Southern blot analyses

Methods

Total genomic DNA was isolated from leaf tissue obtained from R3 and R6 generation plants of GTS 40-3-2 according to Dellaporta *et al.* (1983) with minor modifications. One or two leaflets from the first trifoliate leaf of greenhouse-grown plants was used as source material and an RNase incubation step followed by a phenol/chloroform extraction was added before the final ethanol precipitation. Genomic DNA was quantitated spectrophotometrically and digested with *Hind*III. Digested samples from each plant (5 μg DNA), as well as *Hind*III digested DNA from the parental A5403 line, and *Eco*RI digested PV-GMGT04 plasmid DNA (100 pg) as a positive control, were separated by 0.8% agarose-TAE gel electrophoresis. Separated fragments were transferred onto nylon membrane and probed with ^{32}P-labelled PV-GMGT01 plasmid DNA and subjected to autoradiography (Southern 1975; Sambrook *et al.* 1989).

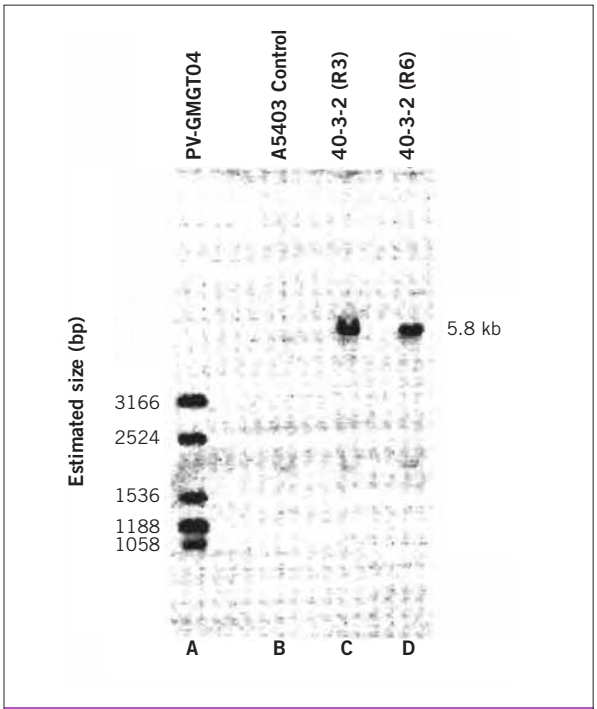


Fig. 18. Southern blot of GTS line 40-3-2 generations R3 and R6 probed with PV-GMGT04. Genomic DNA prepared from generation R3 (lane C) and R6 (lane D) plants of line GTS 40-3-2, as well as the parental non-transgenic A5403 soybean line (lane B), was digested with *Hind*III, separated by electrophoresis and transferred onto nylon membrane that was probed with 32P-labelled PV-GMGT04 plasmid DNA. As a positive control, a sample of *Eco*RI digested PV-GMGT04 DNA was included in lane A.

Results and discussion

Previous analyses using polymerase chain reaction (PCR) amplification with specific 5' and 3' terminal primers had verified the boundary regions of the inserted DNA and demonstrated that neither *Hind*III site originally present in plasmid PV-GMGT04 (at positions 155 and 2707) was incorporated into the host genome. Southern blot analysis of *Hind*III digested genomic DNA from the original transformant had demonstrated the presence of a 5.8 Kb fragment, indicating that the two *Hind*III sites bordering this fragment must be located in the plant genome, on either side of the inserted DNA

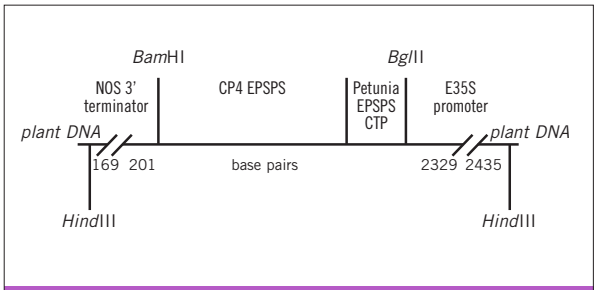


Fig. 19. Diagramatic representation of the insert contained in GTS 40-3-2 showing *Hind*III digestion sites. Based on PCR analysis of the 5' and 3' terminal regions of the inserted DNA fragment, neither *Hind*III site present at positions 155 and 2707 of plasmid PV-GMGT04 were incorporated into the host plant genome. The two *Hind*III sites bordering the 5.8 Kb fragment (Fig. 18) are located in the plant genome.

(Fig. 19). As it contains both inserted and border DNA, this fragment was considered an appropriate sentinel for monitoring the inserted DNA's stability in GTS 40-3-2.

When *Hind*III digested genomic DNA from generation R3 and R6 GTS 40-3-2 plants was probed with 32P-labelled PV-GMGT04, a single 5.8 Kb fragment was detected (Fig. 18). The fact that this same size fragment is present in both generations of 40-3-2 indicates that the plasmid DNA insert and the plant border DNA are stably maintained throughout the plant life cycle over four generations. Similar, more sensitive Southern blot analyses were also able to demonstrate the co-segregation of a second inserted DNA fragment containing a 72 bp sequence corresponding to a region from the CP4 EPSPS encoding gene. These data indicated that the primary insert and this second, smaller insert behaved as a single genetic locus.

Inheritance

Confirmation that the glyphosate tolerance trait present in GTS 40-3-2 segregates according to a defined pattern (Mendelian segregation) was obtained from the analysis of F2 progenies of backcrosses between GTS 40-3-2 and other, non-transgenic, soybean lines.

Table 3 summarizes the segregation patterns of progeny of crosses between 40-3-2 and 17 non-transgenic cultivars. A consistent 3 tolerant to 1 sensitive ratio was observed among all F2 progeny, indicating that the glyphosate tolerance in 40-3-2 is conditioned by a single dominant gene.

Conclusion

The information summarized in this section supports the conclusion that GTS 40-3-2 containing the gene encoding CP4 EPSPS is genetically stable, and that any conclusions regarding the safety of GTS 40-3-2 are also valid for its progeny and other soybean varieties derived from it through classical breeding techniques.

Table 3. Segregation of glyphosate tolerance in F_2 progeny of crosses between GTS 40-3-2 and 17 non-transgenic cultivars

Family	*Tolerant*	*Sensitive*	*Chi²*
1	17	4	0.40
2	10	2	0.44
3	12	4	0.00
4	16	4	0.27
5	16	5	0.02
6	14	3	0.49
7	18	5	0.13
8	10	4	0.10
9	17	7	0.22
10	6	3	0.33
11	15	4	0.16
12	17	1	3.63
13	10	1	1.48
14	16	5	0.02
15	3	1	0.00
16	18	3	1.29
17	19	5	0.22
Total	234	61	2.94

Uncorrected chi-square goodness-of-fit test for hypothesis of 3:1 segregation. None of the chi-square values are significant at the 95% confidence level ($chi2_{0.05}$=3.84).

References

Dellaporta, S.L., Wood, J. & Hicks, J.B. (1983). A plant DNA minipreparation: Version II. *Plant Mol. Biol. Reporter* **1**, 19-21.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Molecular Biology* **98**, 503-517.

Expressed material / effect

Materials and methods

Field trials

In order to generate plant material for expression and quality analysis, field trials were conducted at one site in

Puerto Rico in 1992, in nine sites across the United States during 1992, and at an additional four sites in the United States in 1993. Plots were arranged in randomized complete block designs and consisted of four genotypes: the parental control line A5403, GTS 40-3-2, as well as two additional GTS lines. Samples of leaf tissue and seeds collected from each trial site were used as test materials for determining the expressed levels of CP4 EPSPS by quantitative enzyme linked immunosorbent assay (ELISA).

ELISA assays

Seed and leaf tissue samples from GTS 40-3-2 and control A5403 plants were prepared for ELISA by grinding to a fine powder in liquid nitrogen and resuspending a weighed volume in extraction buffer (100 mM Tris-HCl pH 7.8, 100 mM sodium borate, 5 mM MgCl2, 0.05% v/v Tween 20, and 0.2% sodium ascorbate) at a 1:100 tissue to buffer ratio (30 mg tissue / 3 ml buffer). The suspension was homogenized (30 sec; PT3000 Polytron), centrifuged to remove cell debris, and the supernatant either assayed immediately or stored frozen at minus 80°C. For the CP4 EPSPS ELISA, the double antibody sandwich (primary antibody from goat and secondary antibody from rabbit) was detected with donkey anti-rabbit alkaline phosphatase conjugate followed by development with p-nitrophenyl phosphate (p-NPP). The GUS direct double antibody sandwich ELISA utilized a commercially available rabbit anti-GUS antibody (CLONTECH Laboratories) and its alkaline phosphatase conjugate, with p-NPP development. Quantitation of CP4 EPSPS or GUS in plant samples was accomplished by extrapolation from the logistic curve-fits of the purified mature CP4 EPSPS (*i.e.*, without transit peptide) or GUS standard curves (both standards purified from *E. coli* overexpression strains).

Western immunoblot analysis

Samples of soybean tissue and processed soybean fractions were ground to a powder in liquid nitrogen using a mortar and pestle, and resuspended in extraction buffer (100 mM Tris-HCl pH 7.5, 1 mM benzamidine-HCl, 5 mM DTT, 2.5 mM EDTA, 1.0 mM PMSF, 10 mM CHAPS, and 6M guanidine-HCl) at a 1:50 tissue to volume buffer ratio. Samples were homogenized with a Omni-2000 hand held homogenizer (setting 4-5; 30 sec), centrifuged to remove cell debris, and the supernatant saved for subsequent analysis. Proteins were separated by SDS-PAGE on pre-cast 4-20% linear polyacrylamide gradient gels using the buffer system of Laemmli (1970). Separated proteins were then electrophetically transferred onto PVDF membrane, treated with Tris buffered saline containing 5% non-fat dried milk powder and 0.2% Tween-20 to block non-specific protein binding sites. CP4 EPSPS protein bound to the membrane was probed using a 1:1000 dilution of goat anti-CP4 EPSPS IgG (1-2 hr at room temperature), and bound antibody was detected by incubating sequentially with biotin-labelled Protein G and horseradish peroxidase-conjugated NeutrAvidin, followed by enhanced chemiluminescence development.

CP4 EPSPS and GUS enzymatic assays

The procedure used to determine the amount of functionally active CP4 EPSPS was based on measuring the incorporation of ^{14}C into EPSPS from ^{14}C-phosphoenol pyruvate (PEP) using high pressure liquid chromatography (HPLC) separation and a radioactivity detector (Padgette *et al.* 1988; Padgette *et al.* 1987). Reactions were incubated at 25C in buffer containing 50 mM HEPES pH 7.0, 0.1 mM ammonium molybdate, 5 mM KF, 1 mM 14C-PEP, and 2 mM shikimate-3-phosphate. For analysis, samples were quenched with 100 mM Tris-HCl pH 7.8, 100 mM sodium borate, 5 mM $MgCL_2$, 0.2% sodium ascorbate, desalted using a disposable spin-column, and separated via HPLC. One unit (U) of enzyme activity was defined a 1 micromole EPSPS produced / minute at 25°C.

The enzymatic assay for GUS was a modification of the method of Jefferson *et al.* (1986), and was based on the GUS-catalyzed formation of p-nitrophenol from p-nitrophenol-beta-D-glucuronide. Reaction mixtures (8 mM p-nitrophenyl-beta-D-glucuronide, 49 mM sodium phosphate, 10 mM 2-mercaptoethanol, 10 mM EDTA, 0.1% sarkosyl, and 0.1% Triton X-100, pH 7.4) were incubated for 1 – 5 min, quenched by the addition of 2.5 M 2-amino-2-methyl-1,3-propanediol, and the production of p-nitrophenol determined spectrophotometrically by measuring the absorbance at 406 nm. One unit (U) of enzyme activity was defined as 1 micromole p-nitrophenol produced / min at 37°C.

Results and discussion

Expression tests for CP4 EPSPS and GUS were performed by ELISA, and, as illustrated in Table 4, only CP4 EPSPS was detectable in either seed or leaf tissue. The mean expression levels of CP4 EPSPS were 0.288 μg/mg tissue (fresh weight) or 0.443 μg/mg tissue, respectively, for seed or leaf tissue collected from field trials during 1992. Similar, but somewhat lower levels of

Table 4. ELISA analysis of CP4 EPSPS and GUS in GTS line 40-3-2

		Ug protein / mg tissue fresh weight	
***Sample*[1]**	***No. of sites***	***Mean***	***Range*[2]**
CP4 EPSPS[3]			
Leaf[4] 1992	8	0.443	0.251-0.789
Leaf[4] 1993	3	0.415	0.299-0.601
Seed 1992	9	0.288	0.186-0.395
Seed 1993	4	0.201	0.127-0.277
GUS[3]			
Leaf[4] 1992	8	ND#	–
Seed 1992	9	ND#	–

1 *All samples were frozen immediately and shipped and stored frozen. Means reported are of the site means. Soybean plant samples for ELISA were generated from nine locations in 1992 and four locations in 1993*

2 *Range denotes the lowest and highest individual assay for each plot.*

3 *No CP4 EPSPS or GUS proteins were detected in the A5403 parental control line samples (grown at identical locations) in either leaf or seed samples.*

4 *The center leaflet from the fully expanded third trifoliate of six plants randomly selected from different rows in various locations in each treatment plot were collected and pooled by plot.*

#ND. Not detected.

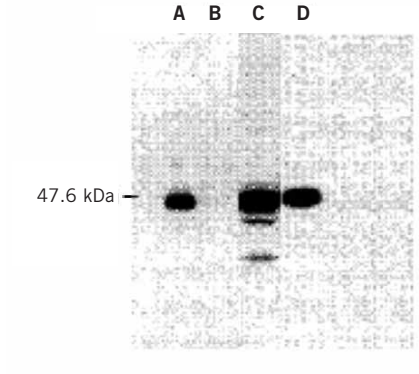


Fig. 20. Western immunoblot detection of CP4 EPSPS protein in samples of GTS 40-3-2 soybean seed (lane C) or toasted meal prepared from GTS 40-3-2 soybean seed (lane D). Purified CP4 EPSPS from an *E. coli* overexpression culture was included as a positive control (lane A), and a negative buffer control sample is shown in lane B.

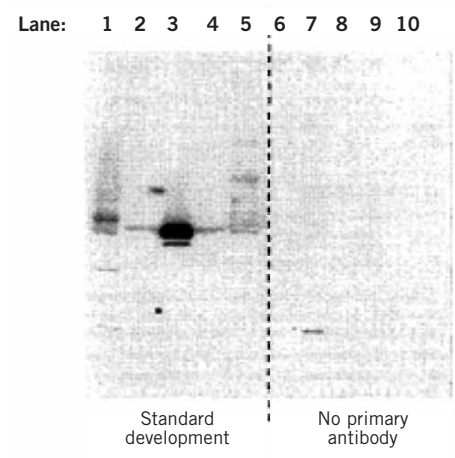


Fig. 21. Specificity of the CP4 EPSPS Western blot analytical method. All EPSPS proteins were expressed in *E. coli* and purified to near homogeneity, and the maize and petunia EPSPS proteins were loaded at 10 times the level of CP4 EPSPS. Samples tested were petunia EPSPS (50 ng; lanes 2, 8), CP4 EPSPS (5 ng; lanes 3, 9), and maize EPSPS (50 ng; lanes 4, 10). Molecular weight markers included the Promega midrange markers (lanes 1, 7) and high range colour markers (lanes 5,6; Amersham). Separated proteins were electroblotted onto PVDF membrane and either processed normally (Standard Development) or left untreated with primary antibody and otherwise processed according the standard procedure (No primary antibody).

expression, were measured for tissue samples collected from four field trials during 1993 (Table 4).

The ELISA results were supported by enzymatic activity assays performed on seed pools of line GTS 40-3-2 collected from the 1992 field tests. The measured glyphosate-tolerant EPSPS activity was 0.025 U/mg but no GUS enzymatic activity was detected. Neither EPSPS nor GUS enzymatic activity was detectable in seed extracts from the non-transgenic parental A5403 soybean line. The lack of detectable GUS protein or enzyme activity confirm Southern blot analyses demonstrating that GUS encoding sequences were not incorporated into the GTS 40-3-2 genome.

Western blot analysis showed that the 47 kDa CP4 EPSPS protein and no additional CP4 EPSPS immunoreactive proteins are detected in event GTS 40-3-2 (Fig. 20). The anti-CP4 EPSPS antisera used for Western blot detection showed almost no cross-reactivity with similar EPSPS proteins derived from different plant sources (Fig. 21).

References

Jefferson, R.A., Burgess, S.M. & Hirsch, D. (1986). Beta-glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* **83**, 8447-8451.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227**, 680-685.

Padgette, S.R., Huynk, Q.K., Aykent, S., Sammons, R.D., Sikorski, J.A. & Kishore, G.M. (1988).

Identification of the reactive cysteines of *Escherichia coli* 5-enolpyruvlshikimate-3-phosphate synthase and their nonessentiality for enzymatic catalysis. *J. Biological Chemistry* **263**, 1798-1802.

Padgette, S.R., Huynh, Q.K., Borgmeyer, J., Shah, D.M., Brand, L.A., Re, D., Bishop, B.F., Rogers, S.G, Fraley, R.T. & Kishore, G.M. (1987). Bacterial expression and isolation of Petunia hybrida 5-enolpyruvylshikimate-3-phosphate synthase. *Arch. Biochem. Biophys.* **258**, 564-573.

Assessment of possible toxicity

Due to the relatively low level of expression of CP4 EPSPS protein in GTS 40-3-2, purified CP4 EPSPS from bacterial cultures was used as test material for the acute mouse gavage and protein digestibility studies described below. This is a common practice when assessing the potential toxicity of introduced novel proteins and requires that physiochemical and functional equivalence be established between bacterial and plant expressed forms of the protein. In the case of *E. coli* expressed CP4 EPSPS (lacking the chloroplast transit peptide), functional equivalence with the plant expressed protein was based on the criteria of molecular weight, immunological cross-reactivity, absence of glycosylation, N-terminal amino acid sequence, and enzymatic activity (Table 6).

Acute mouse gavage study with CP4 EPSPS protein

Methods

An acute mouse gavage study using *E. coli* produced mature CP4 EPSPS protein (lacking the chloroplast transit peptide) was performed to directly assess the potential toxicity associated with the CP4 EPSPS protein (Naylor 1993). CP4 EPSPS protein was administered by oral gavage at dosages up to 572 mg/kg of body weight. Mice were observed twice daily for signs of toxicity and food consumption was recorded daily. Food and water were provided ad libitum. All animals were sacrificed on post-dosing day 8 and 9 and subjected to gross necropsy. Approximately 40 tissues were collected and saved from each animal in the test.

Results and discussion

The results from this study demonstrated that there were no adverse effects on mice administered the CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg. The dose represented an approximate 1300-fold safety margin relative to the highest potential human consumption of plant-expressed CP4 EPSPS, assuming no loss of protein due to processing. There were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and CP4 EPSPS protein-treated groups.

Digestion of CP4 EPSPS in simulated gastric and intestinal fluids

Methods

Simulated mammalian gastric and intestinal digestive fluids were used in in vitro assays to assess the susceptibility of *E. coli* expressed CP4 EPSPS to proteolytic degradation. Simulated gastric and intestinal fluids were prepared as described in the United States Pharmacopeia (US Pharmacopeia 1990), a frequently cited reference for *in vitro* digestion studies. *In vitro*

Table 6. Summary of equivalence analyses: GTS *vs. E. coli* CP4 EPSPS proteins

Analytical Method	*Criteria*	*Results*
SDS-PAGE	Similar electrophoretic mobility.	Similar apparent MW.
Western immunoblot	Similar electrophoretic mobility and immunological response.	Similar apparent MW and immunological response.
Glycosylation	Comparable response with glycosylation detection.	No CP4 EPSPS specific carbohydrate moieties detected.
Amino Acid Sequence	Corresponds through 10 amino acid positions.	Correct N-terminus through 15 positions (N-terminal methionine present on *E. coli* produced CP4 EPSPS).
CP4 EPSPS Enzymatic Activity	Specific activities (SA) will not differ more than a factor of 2.	GTS 3.9 U/mg *E. coli* 3.0 U/mg.
ELISA	Comparable done response.	Dose response curves comparable.

digestive fate of CP4 EPSPS was monitored using Western immunblot analysis and by measuring enzymatic activity of aliquots removed at various times following the start of digestion.

Results and discussion

CP4 EPSPS was rapidly degraded in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) with a half-life of less than 15 seconds or less than 10 minutes, respectively. To put the rapid *in vitro* degradation of the CP4 EPSPS protein into perspective, solid food has been estimated to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes (Sleisenger & Fordtran 1989). If some of the CP4 EPSPS protein did survive the gastric system, it would be rapidly degraded by intestinal proteases. The transit time through the intestine (for 51Cr-labelled chromate, which is not absorbed) has been estimated to be 4-10 hours for the first products to appear in the feces and 68-165 hours for the last to be detected. Thus the T_{50} of 10 minutes for the in vitro degradation of CP4 EPSPS provides a wide margin of assurance that virtually all of the protein would be degraded during its initial transit through the intestinal tract.

Lack of homology of CP4 EPSPS protein with other protein toxins

The deduced (predicted) amino acid sequence of the CP4 EPSPS was compared with the sequences of 1935 known protein toxins present in the Pir protein, Swissprot, and Genpept protein databases. The analysis of homology of CP4 EPSPS protein to known protein toxins was based on the fact that patterns of amino acid sequence or regions of strong homology shared between two or more proteins may provide insight into the biological activity of the protein. Homologous proteins derived from a common ancestor have similar amino acid sequences, are structurally similar and often share common function. Homology was determined by comparing the degree of amino acid sequence similarity between proteins using published criteria (Doolittle 1990). There were no detected homologies with known toxins. The lack of significance between the alignments was assessed by randomizing the CP4 EPSPS amino acid sequence, keeping relative proportions of individual amino acids the same, and comparing the randomized sequence with the identical database of known protein toxins. The output comparisons generated in this manner closely resembled the results obtained with the unrandomized CP4 EPSPS sequence.

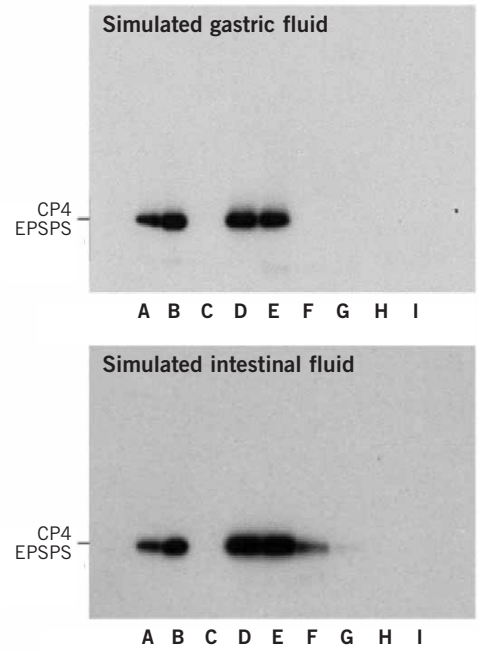


Fig. 33. *In vitro* digestibility of *E. coli* expressed CP4 EPSPS in either simulated gastric fluid (top panel) or simulated intestinal fluid (bottom panel). Aliquots were removed at 0, 15, 30, 60, and 120 seconds after the start of digestion with SGF (lanes E through I, top panel), or at 0, 10, 32, 100, and 270 minutes after the start of digestion with SIF (lanes E through I, bottom panel) and subjected to SDS-PAGE. Separated proteins were electroblotted onto PVDF membrane and treated sequentially with rabbit anti-CP4 EPSPS IgG and 125IProtein G. Samples of purified CP4 EPSPS (5, 10 ng in lanes A, B, respectively), buffer control (lane C), and CP4 EPSPS in reaction buffer w/o digestive enzymes (lane D) were included on each gel.

Conclusion

In summary, the CP4 EPSPS protein shows no amino acid sequence similarity to known protein toxins, is rapidly degraded in vitro under conditions simulating the digestive conditions in the mammalian stomach or intestinal tract, and displays no indications of acute toxicity as measured by treatment-related adverse effects in mice administered CP4 EPSPS protein by oral gavage.

References

Doerfler, W. & Schubbert, R. (1997). Fremde DNA im Saugersystem. *Deutsches Arzteblatt* **94**, 51-52.

Doolittle, R.F. (1990). Searching through sequence databases. *In*: Molecular Evolution: Computer Analysis of Protein and Nucleic Acid Sequences. Doolittle, R.F. (ed.). Pp. 99-110. Academic Press, San Diego, CA.

Naylor, M. (1993). Acute oral toxicity study of CP4 EPSPS in albino mice. Monsanto Report ML92542, St. Louis, MO.

OECD (2000). Report of the task force for the safety of novel foods and feeds. C(2000)86/ADD1 Organization for Economic Cooperation and Development, Paris.

Sleisenger, M.H. & Fordtran, J.S. (1989). Gastrointestinal Disease. Volume 1, Pathophysiology Diagnosis Management. 4th Edition. W.B. Saunders Co., Toronto. pp 685-689.

US Pharmacopeia (1990). Vol. XXI, NF XVII. United States Pharmacopeial Convention, Inc., Rockville, MD. 1788 pp.

Assessment of possible allergenicity

The potential allergenicity of the CP4 EPSPS protein expressed in transgenic GTS 40-3-2 soybeans was assessed by examining: (1) the immunoreactivity of separated soybean proteins with IgE antibodies from sera obtained from soybean allergic individuals; (2) the physiochemical properties of CP4 EPSPS in relation to known allergenic proteins; (3) the lability of CP4 EPSPS in simulated gastric and intestinal fluids; (4) amino acid sequence similarities with other naturally occurring plant derived EPSPS enzymes and with known protein allergens; and (5) estimated dietary exposure to CP4 EPSPS based on its concentration in food.

Immunoreactivity with sera from sensitized individuals

Protein extracts were prepared from non-toasted, defatted soy flour derived from GTS 40-3-2, the parental A5403 line, and three commercially available soy flour preparations, and separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Separated proteins were electroblotted onto PVDF membranes and probed with IgE antibodies from pooled serum obtained from several individuals shown to be sensitive to soybean products by direct food challenges (Burks *et al.* 1988). As controls, IgE antibodies from normal and peanut-sensitive individuals were used to test the specificity of similar antibodies from soybean-sensitive individuals.

Both the presence and the relative levels of the endogenous allergenic proteins in all of these soybean preparations were comparable, demonstrating that the profile of allergenic proteins was not significantly altered during the production of GTS 40-3-2.

Physiochemical properties of CP4 EPSPS

Although the molecular mass of CP4 EPSPS, 47.6 kDa, is within the size range of 10-70 kDa reported for many allergenic proteins, its other physiochemical properties are not consistent with the characteristics of most allergenic proteins. CP4 EPSPS is not heat stable and all detectable enzymatic activity and tertiary structure are lost (established by loss of ELISA reactivity) after the toasting step during processing (Padgette *et al.* 1993). This instability of CP4 EPSPS during processing was expected based on the rapid loss of activity observed with the purified protein upon heat treatment (65C, 15 minutes).

As most protein allergens are glycosylated, the plant-expressed CP4 EPSPS protein was examined for the presence of carbohydrate moieties, and found not to be glycosylated (Harrison *et al.* 1993). This result was expected since protein glycosylation requires passage through the rough endoplasmic reticulum and Golgi bodies, which requires specific targeting sequences on the N-terminus of the protein that were not engineered into the CP4 EPSPS construct. The CP4 EPSPS gene product was targeted to the chloroplast, the site of aromatic amino acid biosynthesis, and this targeting does not require or enable glycosylation.

Stability to *in vitro* digestion

The ability of food allergens to reach and to cross the mucosal membrane of the intestine, and thus enter the circulatory system, is a likely prerequisite to allergenicity. A protein that is stable to the acid-protease and proteolytic conditions of the stomach and intestine, respectively, has an increased probability of reaching the intestinal mucosa. Many allergenic proteins exhibit proteolytic stability (King *et al.* 1967; Kortekangas-Savolainen *et al.* 1993; Onaderra *et al.* 1994; Taylor 1992; Taylor *et al.* 1987; Metcalfe 1985), although the majority remain untested.

As has already been discussed in Chapter 9 (Toxicity), the CP4 EPSPS protein was extremely

susceptible to degradation (Ream *et al.* 1993) in both simulated gastric fluids (*e.g.*, pepsin digestion; T50 < 15 seconds) and simulated intestinal fluids (*e.g.*, trypsin digestion; T50 < 10 minutes). This lability to digestion by proteases present in the mammalian digestive tract is not a feature of most protein allergens, and provides additional evidence supporting the lack of allergenic potential for CP4 EPSPS.

Amino acid sequence analysis

The predicted amino acid sequence of the CP4 EPSPS protein was compared with the amino acid sequences of 121 known allergenic proteins contained in three protein databases (Genpept, Pir protein, and Swissprot) using the FASTA computer program (Pearson & Lipman 1988). No biologically significant homology (Doolittle 1990) and, based on an epitope size of 8 contiguous amino acids, no immunologically significant sequence similarities were observed with allergens.

Prevalence in food

A significant factor contributing to the allergenic potential of food proteins is their concentration in foods. Most allergens are present as major protein components in the specific food, in amounts ranging from 1-80% of the total protein (Fuchs & Astwood 1996). This is true for the allergens in milk (Taylor *et al.* 1987), soybean (Burks *et al.* 1988), and peanuts (Barnett *et al.* 1983). In contrast, the CP4 EPSPS is present in very low levels in soybean seed (0.03% fresh weight, or 0.08% of the total protein).

Conclusion

In summary, the data and analyses described above and summarized in Table 7 support the conclusion that the CP4 EPSPS protein is not derived from an allergenic source, does not possess immunologically relevant sequence similarity with known allergens, and does not possess the characteristics of known protein allergens. This information, coupled with the extremely rapid digestion of this protein under in vitro digestive conditions that mimic human digestion, established that there is no reason to believe that plant expressed CP4 EPSPS protein should pose any significant allergenic risk for consumption of the products generated from GTS 40-3-2 soybeans.

Table 7. Characteristics of known protein allergens[1]

Characteristic	*Allergens*	*CP4 EPSPS*
Allergenic source of gene	yes	no
Mol wt 10-70 kDa	yes	yes
Glycosylated	yes[2]	no
Similar sequence to allergens	yes	no
Stable to digestion	yes	no
Stable to processing	yes	no
Prevalent protein in food	yes	no

1. As described in Taylor (1992) and Taylor et al. *(1987).*
2. Typically, but not absolutely.

References

Anderson, J.A. (1996). Allergic reactions to foods. *Critical Reviews in Food Science and Nutrition* **36**, S19-S38.

Barnett, D., Baldo, B.A. & Howden, M.E.H. (1983). Multiplicity of allergens in peanuts. *J. Allergy Clin. Immunol.* **72**, 61-68.

Bock, S. A. (1987). Prospective appraisal of complaints of adverse reactions to foods in children during the first three years of life. *Paediatrics* **79**, 683-688.

Burks, A.W., Brooks, J.R. & Sampson, H.A. (1988). Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J. Allergy Clin. Immunol.* **81**, 1135-1142.

Burks, A.W. & Sampson, H. (1993). Food allergies in children. *Current Problems in Paediatrics* **23**, 230-252.

Doolittle, R.F. (1990). Searching through sequence databases. *Methods in Enzymology* **183**, 99-110.

Fuchs, R.L. & Astwood, J.D. (1996). Allergenicity assessment of foods derived from genetically modified plants. *Food Technology* **50**, 83-88.

Harrison, L.A., Bailey, M.R., Leimgruber, R.M., Smith, C.E., Nida, D.L., Taylor, M.L. & Padgette, S.R. (1993). Equivalence of plant- and microbially-expressed proteins: CP4 EPSPS from glyphosate-tolerant soybeans and *E. coli.* Monsanto Study 92-01-30-11, Monsanto Technical Report MSL-12899, St. Louis, MO.

Hefle, S.L., Nordlee, J. A. & Taylor, S. L. (1996): Allergenic foods. *Critical Reviews in Food Science and Nutrition* **36**, S69-S89.

King, T.P., Norman, P.S. & Connell, J.J. (1967). Isolation and characterization of allergens from ragweed pollen, IV. *Biochemistry* **6**, 1992-2000.

Kortekangas-Savolainen, O., Savolainen, J. & Einarsson, R. (1993). Gastrointestinal stability of baker's yeast allergens: an *in vitro* study. *Clin. Exp. Allergy* **23**, 587-590.

Mekori, Y. A. (1996). Introduction to allergic disease. *Critical Reviews in Food Science and Nutrition* **36**, S1-S18.

Metcalfe, D.D. (1985). Food allergens. *Clin. Rev. Allergy* **3**, 331-349.

Metcalfe, D.D., Astwood, J. D., Townsend, R., Sampson, H. A., Taylor, S. L. & Fuchs, R. L. (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* **36**, S165-S186.

Onaderra, M., Monsalve, R.I., Mancheno, J.M., Villalba, M., Martinez Del Pozo, A., Gavilanes, G. & Rodriguez, R. (1994). Food mustard allergen interaction with phospholipid vesicles. *Eur. J. of Biochem.* **225**, 609-615.

Padgette, S.R., Nida, D.L., Biest, N.A., Bailey, M.R. & Zobel, J.F. (1993). Glyphosate tolerant soybeans in the U.S. in 1992: Field test, processing studies, and analytical evaluation. Monsanto Study 92-01-30-02, Technical Report MSL-12906, St. Louis, MO.

Parker, S. L., Leznoff, A., Sussman, G. L., Tarlo, S. M. & Krondl, M. (1990). Characteristics of patients with food-related complaints. *Journal of Allergy and Clinical Immunology* **86**, 503-511.

Pearson, W. & Lipman, D. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**, 2444-2448.

Ream, J.E., Bailey, M.R., Leach, J.N. & Padgette, S.R. (1993). Assessment of the in vitro digestive fate of CP4 EPSP synthase. Monsanto Study 92-01-30-15, Technical Report MSL-12949, St. Louis, MO.

Sampson, H. A. (1990). Immunologic mechanisms in adverse reactions to foods. *Immunology and Allergy Clinics of North America* **11**, 701-706.

Sampson, H. A. & Burkes, A. W. (1996). Mechanisms of food allergy. *Annual Review of Nutrition* **16**, 161-177.

Taylor, S.L. (1992). Chemistry and detection of food allergens. *Food Technol.* **39**, 146-152.

Taylor, S.L., Lemanske Jr., R.F., Bush, R.K. & Busse, W.W. (1987). Food allergens: Structure and immunologic properties. *Ann. Allergy* **59**, 93-99.

Compositional analyses of key components, evaluation of metabolites, food processing and nutritional modification

Nutrition data were obtained from analyses of glyphosate-tolerant and control soybeans (parental variety A5403) grown at nine field locations in 1992. These sites were chosen to be representative of the wide geographical area in which soybeans are grown. In addition, a four-site field test with limited analytical evaluations was performed in 1993. As the emphasis of these analyses was to examine any effects of the introduced gene and protein, the test material was derived from soybeans that had not been treated with glyphosate herbicide.

Although many of the analyses were performed on soybean seed, several soy protein products were also manufactured from GTS 40-3-2 for additional testing. Toasted meal was chosen because it is the main soybean protein product used in animal feed, defatted meal (flour) was prepared because it is the starting material for a large number of soybean products used in food, and protein concentrate from defatted meal was also evaluated because of its food use. In addition, crude lecithin and refined, bleached deodorized oil were manufactured.

Proximate analysis

Compositional (proximate) analyses were performed on soybean seeds derived from GTS 40-3-2 and the parental non-transgenic control line, A5403. The concentrations of carbohydrate, protein, fat, moisture, fibre, and ash, expressed on a dry-weight basis, were measured according to published procedures of the Association of Official Analytical Chemists (AOAC).

Methods

Ash

Volatile organic matter was driven off when the sample was ignited at 550°C in an electric furnace. The residue was quantitated gravimetrically and calculated to determine percent ash (AOAC method 923.03, 1990). Using a 3 g sample, the lowest confidence level of this method was 0.2%.

Carbohydrates

Carbohydrates were calculated by difference using the fresh weight-derived data and the following equation (USDA Agricultural Handbook No. 8, 1975):

% carbohydrates =
100% - (% protein + % fat + % ash + % moisture)

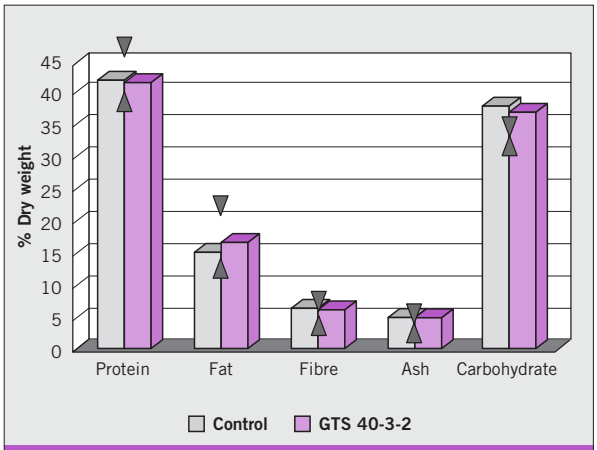


Fig. 22. Proximate analysis of soybean seeds. Bars represent the means of seeds from nine field sites, and the triangles represent the high and low values reported in the literature for each respective component.

Fig. 23. Proximate analysis of soybean seeds. Bars represent the means of seeds collected from four field sites in 1993, and the triangles represent the high and low values reported in the literature for each respective component.
Similar analyses performed on samples of toasted (Fig. 24) and non-toasted meal, and protein concentrate prepared from GTS 40-3-2 and control non-transgenic soybeans did not reveal any appreciable differences in the levels of macronutrients.

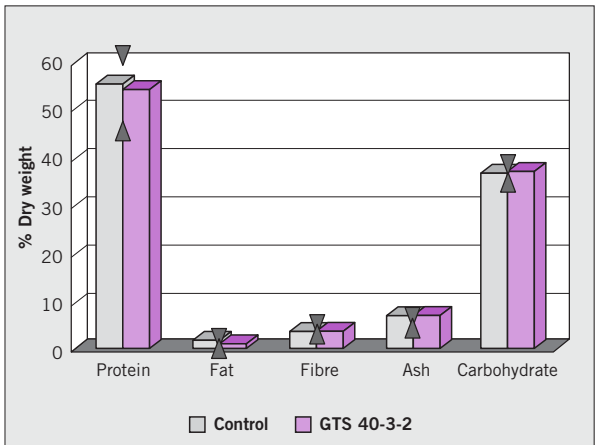


Fig. 24. Proximate analysis of toasted meal. Bars represent the means of three processing studies, and the triangles represent the high and low values from the literature for each respective component.

Crude Fibre

Crude fibre is the loss on ignition of dried residue remaining after digestion of the samples with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions under specific conditions (AOAC method 7.066-7.070, 1984). Using a 2 g sample, the lowest confidence level of this method was 0.2%.

Fat

The fat was extracted using ether and hexane. The extract was washed with a dilute alkali solution and filtered through a sodium sulfate column. The remaining extract was evaporated, dried and weighed (AOAC methods 920.39C). Using a 2 g sample, the lowest confidence level of this method was 0.1% fat.

Moisture

The sample was dried to a constant weight in a vacuum oven at 133°C (approximately 2 hours) (AOAC method 44-15A, 1987). The moisture loss was determined gravimetrically.

Protein

Protein and other organic nitrogen in the sample were converted to ammonium sulfate by digesting the sample with sulfuric acid containing a potassium sulfate/titanium dioxide/cupric sulfate catalyst mixture. The acid digest was made alkaline, and the ammonia was distilled and titrated with standard acid. The percent nitrogen was determined and converted to protein using the factor 6.25 (AOAC method 988.05, 1990). Using a 1 g sample, the lowest confidence level of this method was 0.1% protein (0.02% nitrogen).

Results

Compositional analyses of protein, fat, fibre, ash, and carbohydrate of GTS 40-3-2 and control soybean seeds obtained from nine field trial sites in 1992 and four trial sites in 1993 are presented in Figures 22 and 23, respectively. For each of the components measured, there were no statistically significant differences between GTS 40-3-2 and control soybeans, and with the exception of total carbohydrate, the measured values were within the range reported in the scientific literature. For the nine-

site study, the mean GTS 40-3-2 seed carbohydrate content was 37.1% dry weight, compared to a literature high of 34%. This difference was not judged as significant from a safety perspective as the mean carbohydrate concentration measured in control soybeans harvested from the same sites was 38.1% dry weight.

Similar analyses performed on samples of toasted (Fig. 24) and non-toasted meal, and protein concentrate prepared from GTS 40-3-2 and control non-transgenic soybeans did not reveal any appreciable differences in the levels of macronutrients.

Amino acid composition

Methods

Seed samples were subjected to acid hydrolysis using 6N HCl, then adjusted to pH 2.2 and the individual amino acids were quantitated using an automated amino acid analyzer equipped with post-column ninhydrin derivatization and colorimetric detection (Moore & Stein 1954).

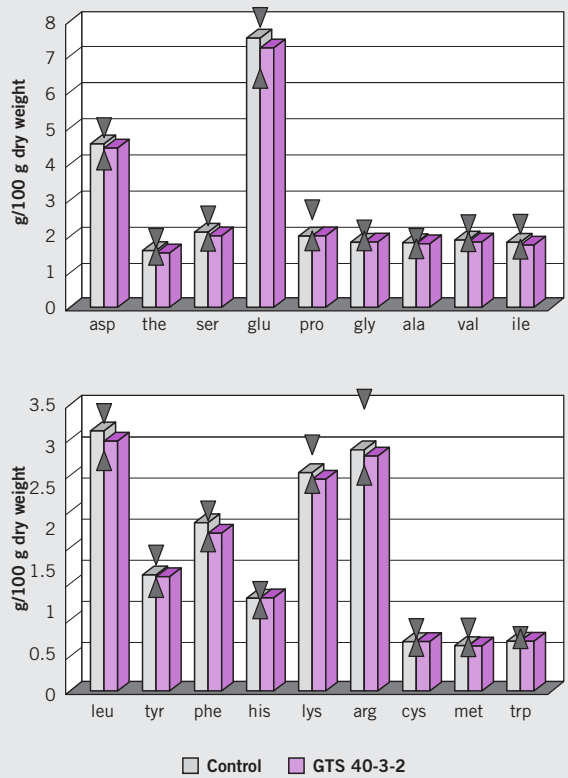


Fig. 25. Amino acid analysis of soybean seeds. Bars represent the mean concentrations of individual amino acids present in samples from soybean seeds harvested from nine field trials during 1992. The triangles represent the high and low values reported in the literature. Several literature values were calculated by converting g amino acid / 100 g protein to g amino acid / 100 g sample by using the mean protein concentration of the seeds analyzed, 41.5%.

Results

For the 18 amino acids measured, there were no statistically significant differences in the levels of any amino acid, including aromatic amino acids, between GTS 40-3-2 seeds and control non-transgenic soybean seeds.

The shikimate pathway plays a central role in plant metabolism and it has been estimated that about one-fifth of the carbon fixed by plants is subsequently channelled through this pathway (Haslam 1993). The lack of any difference in the levels of aromatic amino acids between transgenic GTS soybean seeds and non-transgenic seeds is supported by the fact that all available evidence suggests that EPSPS is not a rate-limiting step in the shikimate pathway, but that regulation of this pathway occurs at the first step in the conversion of erythrose 4-phosphate to 2-keto-3-deoxy-D-arabinoheptulosonate 7-phospate (DAHP) by DAPH synthase (Weiss & Edwards 1980). Increased EPSPS activity would not, therefore, be expected to increase the levels of aromatic compounds in plants, and it has been observed that plant cells expressing 40-times more EPSPS than wild-type cultures do not overproduce aromatic amino acids (Smart *et al.* 1985).

Fatty acid composition

Methods

Samples of soybean seed or refined soybean oil were extracted with chloroform/methanol, saponified with alcoholic potassium hydroxide, and the free fatty acids were then extracted with hexane, washed with water and dried with sodium sulfate. Fatty acids were esterified with methanol, using boron trifluoride as a catalyst, taken up in heptane and subjected to gas chromatographic analysis (AOAC method 983.23 1990). The percent abundance of individual fatty acid methyl esters was calculated relative to the total amount of fatty acid methyl esters present. The lowest confidence level of this method was 0.1% of an individual fatty acid methyl ester.

Results

The relative abundances of individual fatty acids were determined for samples of soybean seed and refined,

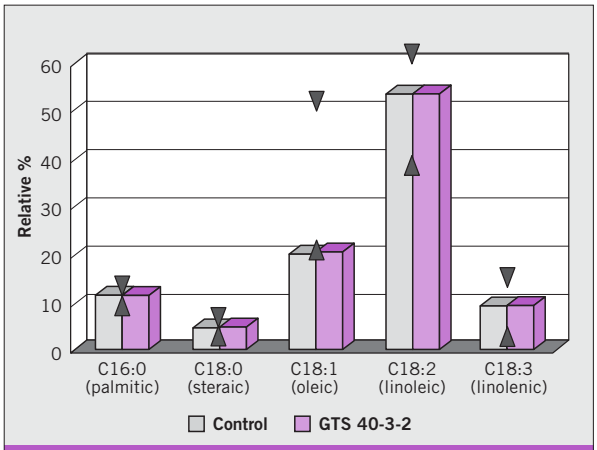


Fig. 26. Fatty acid analysis of soybean seeds. Bars represent the mean levels of individual fatty acids determined from seeds from nine field trial sites in the United States in 1992. The triangles represent the high and low values from the literature for each respective fatty acid.

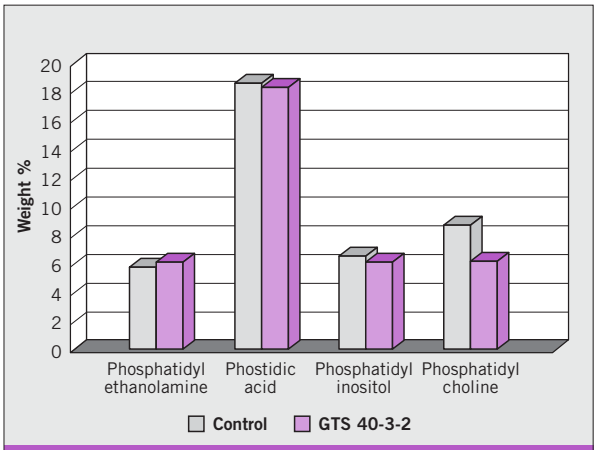


Fig. 27. Crude lecithin analysis of refined, bleached, deodorized soybean oil prepared from soybean seeds harvested from 4 field trial locations in the United States in 1993. Literature values were not available for the components of this crude lecithin fraction.

bleached, deodorized oil derived from GTS 40-3-2 and control non-transgenic soybeans (Fig. 26). There was only one statistically significant difference in the seed fatty acid composition between GTS 40-3-2 and control soybeans; this was for C22:0 fatty acids, which represent less than 0.6% of the total fatty acid fraction. All values, even those for C22:0 from seeds, were within the normal range of values for each respective fatty acid as reported in the literature.

Lecithin, which is a phosphatide removed from crude soybean oil, is used as a natural emulsifier, lubricant, and stabilizing agent (Waggle & Kolar, 1979). In addition to analysis of the free fatty acid profile of refined, bleached, deodorized soybean oil prepared from GTS 40-3-2 and non-transgenic soybeans, these oil samples were used to prepare crude lecithin fractions that were analyzed for phosphatide composition (phosphatidyl ethanolamine, phostidic acid, phosphatidyl inositol, phosphatidyl choline) (AOAC method Ja 7b-91). The relative abundance of each of these phosphatide components was comparable between crude lecithin fractions prepared from GTS 40-3-3 soybean oil and control non-transgenic soybean oil.

Soybean seed proteins

The profiles of seed storage proteins extracted from GTS 40-3-2 and control non-transgenic soybean seeds were compared by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). There were no discernable differences between transgenic and control soybeans (Fig. 28), which indicates that the gross

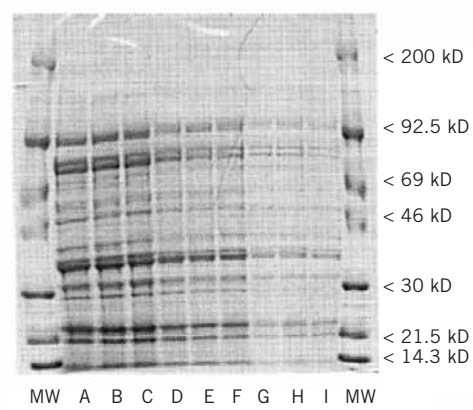


Fig. 28. Coomassie blue stained SDS-PAGE of soybean seed proteins. Composite seed samples from GTS 40-3-2 (lanes B, E, H), control non-transgenic line A5403 (lanes A, D, G), and an additional GTS line 61-67-1 (lanes C, F, I) were extracted, denatured with SDS and 1% 2-mercaptoethanol, and subjected to SDS-PAGE on a 4-20% gradient of polyacrylamide. Aliquots representing 25, 12, or 6.25 ug protein were loaded in each of three lanes, for each soybean sample.

protein compositions of GTS 40-3-2 seeds are not materially different from that of the control soybeans.

Levels of antinutrients

Soybean is naturally a source of several compounds that have been associated with antinutritive effects. These include protease inhibitors, such as soybean trypsin inhibitor, lectins (*e.g.*, soybean hemagglutinin),

isoflavones, and phytate, which complexes with inorganic phosphorous in seed but can also sequester other metallic ions such as iron, calcium, zinc, and magnesium, rendering these elements nutritionally unavailable. The levels of these antinutrient factors were determined in samples of GTS 40-3-2 soybean seed, as well as toasted soybean meal used for livestock feed, and compared with the levels found in the parental non-transgenic soybean line.

Trypsin inhibitors

The antinutritive effect of trypsin inhibitors in unheated soybean products has been the subject of much research (Rackis *et al.*, 1986). The destruction of trypsin inhibitors and consequent elimination of hypertrophic pancreas effects is an important step in the processing of raw soybeans into products with excellent protein quality (Anderson *et al.* 1979).

Trypsin inhibitory activity was measured on alkaline (pH 9.5 - 9.8) extracts of raw soybean seed, or toasted meal, by incubation with a known concentration of trypsin, followed by the addition of benzoyl-D-arginine-p-nitroanilide (BAPNA). Measurements of the absorbance at 410 nm were taken after 10 minutes of reaction. Uninhibited trypsin catalyzes the hydrolysis of BAPNA, forming a yellow-coloured p-nitroaniline. One trypsin unit was defined as an increase equal to 0.01 absorbance units at 410 after 10 minutes per 10 ml reaction volume. The lowest confidence level of this method was 1 trypsin inhibitor unit (TIU) / mg sample, using a 1 g sample.

In comparing extracts of raw soybean seeds from GTS 40-3-2 and non-transgenic control lines (Fig. 29), there were no statistically significant differences in trypsin inhibitor activity. The normal processing of soybean meal to produce toasted meal results in a greater than 90% elimination of trypsin inhibitor activity from both GTS 40-3-2 and control material (Fig. 8.9).

Lectin analysis

Plant lectins are a class of proteins with specific binding affinities for carbohydrate containing glycoproteins that are usually present in plant cell walls and the plasma membrane of cells. The binding of lectins to cell surface glycoproteins may cause agglutination, mitosis, or other biochemical changes in the cell. The ingestion of lectins, such as soybean hemagglutinin, has been associated with a range of antinutritive effects and some disease pathologies. Soybean lectin has been quoted as being responsible for about 25% of the growth inhibition attributable to the ingestion of raw soybean meal by rats (Leiner 1953), although it has since been concluded by some that soybean agglutinin does not play any major role as a determinant of the nutritional quality of soybean protein (Leiner 1980). Other authors still believe that circumstantial evidence exists that soybean lectin may make an appreciable contribution to observed growth inhibition caused by dietary exposure to uncooked soybean meal (Pusztai 1989).

The levels of soybean lectin in raw and toasted soybean meal were estimated by measuring the hemagglutination activity of various extracts against

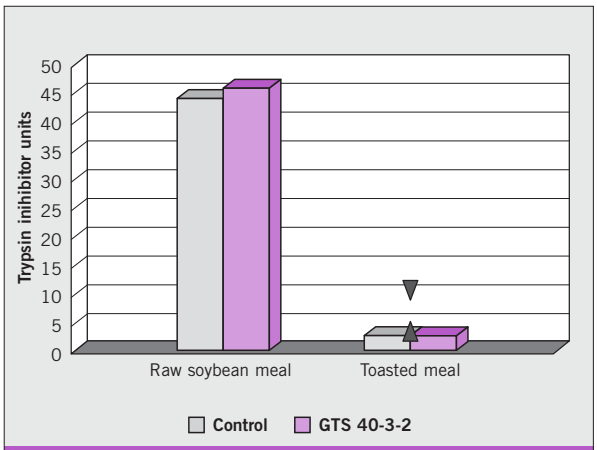


Fig. 29. Trypsin inhibitor activity of raw and toasted soybean meal. Bars represent the results of duplicate studies, and the triangles represent the high and low values for trypsin inhibitor activity reported in the literature for toasted soybean meal.

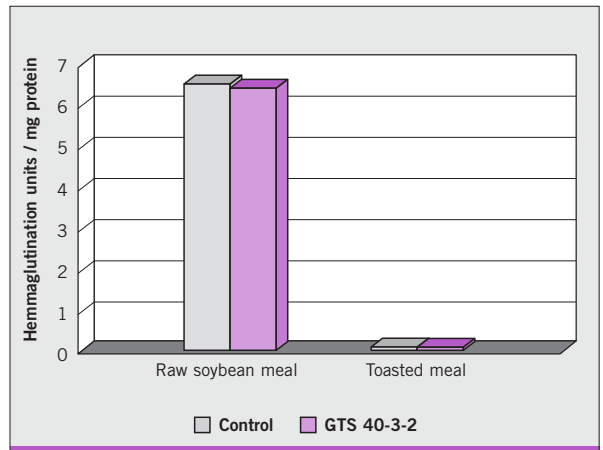


Fig. 30. Soybean lectin analysis of raw and toasted soybean meal. Bars represent the mean values obtained using composite samples of soybeans harvested from nine field trials during 1992. Values are expressed as hemagglutination units (HU) / mg protein.

rabbit red blood cells (Leiner, 1955; Klurfeld & Dritchevski, 1987). There were no statistically significant differences in the lectin activity between GTS 40-3-2 and control non-transgenic soybeans. The level of hemagglutination activity in raw soybean meal was less than 7 hemagglutination units (HU) / mg protein and essentially undetectable in samples of toasted meal (Fig. 30). A comparison of the hemagglutinin activity observed for raw meal in these tests with previously published values of 60-426 HU / mg protein was not informative due to the variability in red cell lots. The sensitivity of the assay was established in positive control tests with purified soybean lectin, in which values of 461-541 HU / mg protein were measured.

Isoflavone analysis

The isoflavones genistein, daidzein, and coumestrol are naturally present in soybeans and their ingestion has been linked to a number of biochemical effects in mammalian species, including estrogenic and hypocholesterolemic activities (Wang *et al.* 1990; Murphy 1982). They have also been reported to contribute to deleterious effects on livestock animals fed soybean meal (Setchell *et al.* 1987).

The bound and free forms of daidzein and genistein were determined in samples of raw and toasted soybean meal by high pressure liquid chromatography (HPLC) separation (Pettersson & Kiessling, 1984). Sample extracts, and extracts following acid hydrolysis to liberate bound isoflavones, were analyzed to calculate the concentrations of free and total isoflavones, respectively. Concentrations of bound isoflavones were calculated as the difference of these two values.

No statistically significant differences in the levels of any isoflavones measured in either raw or toasted soybean meal were detected between GTS 40-3-2 and non-transgenic control soybeans (Fig. 31). The large variability observed in values determined for seeds harvested from different field trial sites was attributed to the effect of environmental variability on the formation of these compounds in plants.

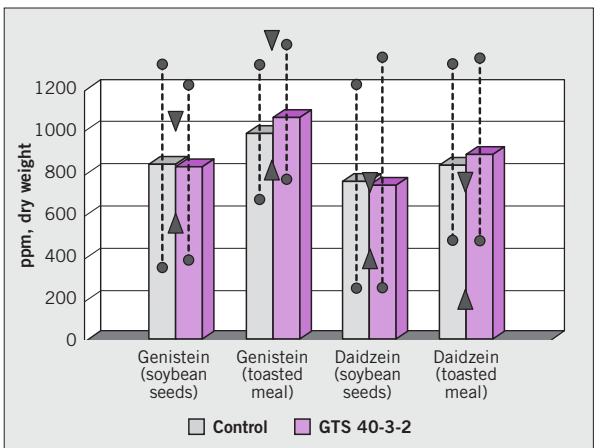


Fig. 31. Genistein and daidzein analysis of soybean seed and toasted meal. For isoflavone levels in soybean seeds, the bars represent the means of values obtained from seed harvested from nine field trial sites in 1992, and in the case of toasted meal, the bars represent the means of three processing studies. The thin lines represent the ranges of experimentally determined values and the literature high and low values in each case are indicated by the triangles.

Stachyose, raffinose, and phytate analysis of soybean meal

The low molecular weight carbohydrates, stachyose and raffinose, are primarily responsible for flatus activity, which is a well known characteristic of soybean products (Rackis 1976). Phytic acid (phytate) is a hexaphosphoric acid derivative of inositol, and exists mainly in soybean seeds as an insoluble, non-nutritionally available calcium-magnesium-potassium complex (Mohamed *et al.* 1991). Phytate is not broken down in monogastric animals (*e.g.*, poultry, fish, swine) and is the main reason that livestock feeds for these animals must be supplemented with additional phosphorus and other minerals, or with phytase enzyme to degrade phytate.

The levels of stachyose and raffinose in extracts prepared from toasted soybean meal were determined by HPLC (Dunmire & Otto 1979). Phytic acid was

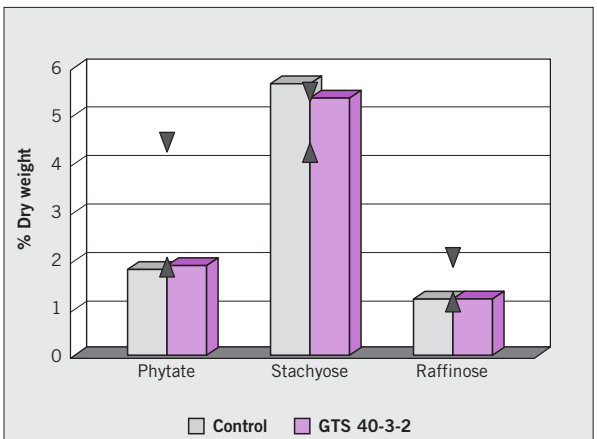


Fig. 32. Phytate, stachyose, and raffinose analysis of toasted meal. Bars represent the means of three processing studies, and the triangles represent the high and low values from the literature for each component.

extracted with dilute HCl and separated from inorganic phosphates by anion exchange chromatography (Ellis & Morris 1983). Bound phytate was eluted with NaCl solution and digested with a mixture of sulfuric and nitric acid to liberate free phosphate, which was quantitated spectrophotometrically following reaction with ammonium molybdate and sulfonic acid. Values were converted to phytic acid based on molecular weight equivalence and the lowest confidence level of the assay was 0.028% phytic acid based on a 2 g sample.

There were no statistically significant differences in the respective levels of stachyose, raffinose, or phytate measured in samples of toasted meal prepared from GTS 40-3-2 or non-transgenic control soybeans (Fig. 32).

Nutrient bioavailability - confirmatory animal feeding studies

In order to establish that the genetic modification resulting in GTS 40-3-2 did not adversely affect the wholesomeness (ability to support typical growth and well-being) of soybean products, animal feeding studies were performed with laboratory rats, broiler chickens, catfish, and dairy cows. Both processed and unprocessed soybean meal was tested on rats because the majority of soybeans used for human food and animal feed are processed by heat treatment, and because rats serve as a surrogate for wild mammals that may eat soybeans in the field. Poultry consume about 49% of the soybeans fed to farm animals and were the subject of a six-week growth study, and dairy cows were included in a four week study since ruminants are normally fed raw soybeans as a source of protein. The catfish study was included since soybean meal is used in diets for commercial aquaculture. Lastly, unprocessed soybean meal was fed for 5 days to bobwhite quail, since birds may feed on soybeans left in the field after harvest.

Methods

Rat Four-Week Feeding Study

Eight week old male and female Charles River CD rats were fed rodent chow containing either processed or unprocessed soybean meal from GTS 40-3-2 or control non-transgenic soybeans for four weeks, ad libitum, at substitution levels of 24.8% or up to 10%, respectively. Feed consumption and body weight were measured at weekly intervals, and rats were observed twice daily for mortality and adverse clinical signs. At the end of the study, all test animals were sacrificed and necropsied. Liver, testes, and kidneys were weighed and approximately 40 tissues were collected and saved from each animal. Dunnett's multiple range comparison test (two-tailed) was used to compare inlife body weights, cumulative body weight gain and food consumption for test and control groups. Terminal body weights, absolute organ weights, and organ/body weight ratios were evaluated by decision-tree statistical analysis procedures to detect group differences and analyze for trends.

Broiler Chicken Six-Week Study

Commercial broiler chicks (White Plymouth Rock x White Cornish; Cobb 500 cockerel x Cobb 500 pullet) were fed test diets containing processed meal from GTS 40-3-2 or the control parental non-transgenic A5403 soybeans, supplemented with corn meal as the only other source of protein. Diets were formulated so as to ensure approximately equal amounts of essential amino acids (methionine, cysteine, lysine, arginine, tryptophan, and threonine), did not contain any medications or growth promoting feed additives, and met the National Research Council requirements for poultry feed. Birds were checked daily for mortality, and any that died on test were removed, weighed and necropsied to determined probable cause of death. Body weights and food consumption were measured, and at the termination of the study, birds were sacrificed and major and minor pectoralis muscles (breast muscles) from the right side were dissected and weighed. Abdominal fat pads were also removed and weighed.

Dairy Cow Four-Week Study

Thirty-six multiparous Holstein dairy cows (93-196 days of lactation) were fed a mixed diet ration (35% alfalfa hay, 17% corn silage, 37% commercial grain mix) containing 10% (w/w dry matter basis) raw soybeans from GTS 40-3-2 or control non-transgenic A5403 soybean lines. This dietary level represented the upper limit for incorporation of raw soybeans into mixed cow diets as fed by dairy farmers, and cows were pre-adapted to high soybean diets prior to the start of the study. Milk samples collected daily during the course of the study were analyzed for lactose, fat, protein, and somatic cells. Total urine and fecal output was collected daily during the last week of the study to determine dry matter digestibility and nitrogen balance.

Catfish Ten-Week Study

Fingerling channel catfish (Ictalurus punctatus), Mississippi Select strain, were maintained for 10 weeks

in glass aquaria and reared on a diet containing soybean meal from GTS 40-3-2 or control non-transgenic soybeans at the same substitution levels used commercially (45-47% w/w). All diets were prepared to contain a final protein concentration of 32%. Fish were weighed at the beginning of the study and on weeks 2, 6, and 10, at which times feed consumption was quantified by subtracting the weight of uneaten pellets removed from the bottoms of tanks from the quantity of feed administered. The cumulative feed conversion ratio was estimated at weeks 2, 6, and 10 by dividing the sum of the feed offered to that point by corresponding total weight gain, adjusting for mortalities. At the end of the study, several fish were selected at random and the edible tissue composited and subjected to proximate analysis.

Table 5. Comparison of feed efficiencies across feeding studies

Line	*Mean Feed Consumption* (g/animal)	*Mean Feed Efficiency*	*Mean Weight Gain* (g)	*Mean Final Weight* (g)
Rat Feeding Study (4 weeks) Processed soybeans				
Males				
Negative control	811	4.58	177[a]	426[a]
A5403 control	764	4.63	165[a,b]	415[a,b]
GTS 40-3-2	749	4.86	154[b]	403[b]
Females				
Negative control	549	8.23	66.7	256
A5403	538	7.87	68.4	259
GTS 40-3-2	538	8.78	61.3	252
Rat Feeding Study (4 weeks) Unprocessed soybeans				
Males				
Negative control	753	6.55	115	431
A5403 5%	755	7.26	104	421
A5403 10%	769	7.25	106	424
GTS 40-3-2 5%	750	7.35	102	420
GTS 40-3-2 10%	768	6.86	112	430
Females				
Negative control	510	12.6	40.6	241
A5403 5%	493	16.3	30.2	231
A5403 10%	513	13.9	36.8	238
GTS 40-3-2 5%	502	13.9	36.2	237
GTS 40-3-2 10%	491	14.2	34.6	236
Broiler Chicken Study (6 weeks) Processed soybeans				
Combined Sex – No statistically significant differences were observed, p<0.05				
A5403 control	3893	1.816	2147	2193
GTS 40-3-2	3844	1.832	2099	2144
Catfish Study (10 weeks) Processed soybeans				
Mixed Sex - No statistically significant differences were observed, p<0.05				
A5403 control	22.1	1.12	19.7	22.6
GTS 40-3-2	21.8	1.17	18.8	21.8

[a], [b]: Means with different letters are statistically different, p<0.05

Line	*Milk* (kg/day)	*Fat* (%)	*3.5% Fat-corrected milk (FCM)* (kg/day)	*Net Energy Intake* (mcal NEL/day)	*FCM/NEL* (kg/mcal)
Dairy Cow Study (4 weeks) Raw, cracked soybeans					
A5403 control	34.9	3.37	34.1[a]	40.1	0.81
GTS 40-3-2	36.2	3.59	36.8[b]	42.9	0.88

[a], [b]: Means with different letters are statistically different, p<0.05

Results

The feed efficiencies (feed conversion ratios) of both GTS 40-3-2 and non-transgenic control soybeans, when used as components of animal feed, were summarized and compared across studies (Table 5). The bobwhite quail study was not included in this comparison because of its short duration (5 days). No statistically significant differences in feed efficiencies were observed when GTS 40-3-2 was used as a feed source compared to the parental variety, A5403. These results were consistent with the extensive compositional analyses demonstrating that GTS 40-3-2 was not significantly different from the control soybeans in terms of its nutritional properties.

References

Anderson, R.L., Rackis, J.J. & Tallent, W.H. (1979). Biologically active substances in soy products. In: Soy Protein and Human Nutrition. H.L. Wilke, D.T. Hopkins & D.H. Waggle, eds. Academic Press, New York. pp. 209-233.

AOAC Method 44-15A. (1987). Moisture-air-oven methods. In AOAC Official Methods of Analysis, Association of Official Analytical Chemists, Arlington, Virginia.

AOAC Method 7.066-7.070. (1984). Fiber (crude) in animal feed, ceramic fiber filter method. In AOAC Official Methods of Analysis, Association of Official Analytical Chemists, Arlington, Virginia.

AOAC Method 920.39C. (1990). Fat (crude) of ether extract in animal feed. In AOAC Official Methods of Analysis, Association of Official Analytical Chemists, Arlington, Virginia.

AOAC Method 923.03. (1990). In Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 983.23. (1990). Fat in foods, chloroform-methanol extraction. In Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia.

AOAC Method 988.05. (1990). Protein (crude) in animal feed, CuSO4/TiO2 mixed catalyst Kjeldahl method. In AOAC Official Methods of Analysis, Association of Official Analytical Chemists, Arlington, Virginia.

AOAC Method Ja 7b-91 (1993). In Official Methods and Recommended Practices of the Association of Official Analytical Chemists, 4th Edition, Arlington, Virginia.

Dunmire, D. & Otto, S. (1979). HPLC determination of sugars in various food products. *Journal of the Official Association of Analytical Chemists* **62**, 176-185.

Ellis, R. & Morris, E.R. (1983). Improved ion-exchange phytate method. *Cereal Chem.* **60**, 121-124.

Haslam, E. (1993). Shikimic acid: Metabolism and metabolites. John Wiley & Sons, Chichester, England.

Klurfeld, D.M. & Kritchevski, D. (1987). Isolation and quantitation of lectins from vegetable oils. *Lipids* **22**, 667-668.

Leiner, I.E. (1953). Soyin, a toxic protein from the soybean. I. Inhibition of rat growth. *J. Nutr.* **49**, 527-540.

Leiner, I.E. (1955). The photometric determination of the hemagglutinating activity of soyin and crude soybean extracts. *Arch. Biochem. Biophys.* **54**, 223-231.

Leiner, I.E. (1980). Anti-nutritional factors as determinants of soybean quality. In: World Soybean Research Conference II: Proceedings. F. Corbin, ed. Westview Press, Boulder, CO. pp. 703-712.

Mohamed, A.I., Mebrahtu, T. & Rangappa, M. (1991). Nutrient composition and anti-nutritional factors in selected vegetable soybean (Glycine max L.). *Plant Foods Hum. Nutr.* **41**, 89-100.

Moore, S. & Stein, W.H. (1954). Procedures for the chromatographic determination of amino acids on four per cent cross-linked sulfonated polystyrene resins. *Journal of Biological Chemistry* **211**, 893-906.

Murphy, P.A. (1982). Phytoestrogen content of processed soybean products. *Food Technology* **36**, 60-64.

Pettersson, H. & Kiessling, K.H. (1984). Liquid chromatographic determination of the plant estrogens coumestrol and isoflavones in animal feed. *Journal of the Official Association of Analytical Chemists* **67**, 503-506.

Pusztai, A. (1989). Lectins. *In*: Toxicants of Plant Origin: Volume II, Proteins and Amino Acids. P.R. Cheeke, ed. CRC Press, Boca Raton, FL. pp. 29-71.

Rackis, J.J. (1976). Flatulence problems associated with soy products. *In*: World Soybean Research. L.D. Hill, ed. The Interstate Printers and Publishers, Inc., Danville, IL. pp. 892-903.

Rackis, J.J., Wolf, W.J. & Baker, E.C. (1986). Protease inhibitors in plant foods: Content and inactivation. In: Nutritional and Toxicological Significance of

Enzyme Inhibitors in Food. M. Friedman, ed. Plenum Press, New York. pp. 299-347.

Setchell, K.D.R., Gosselin, S.J., Welsh, M.B., Johnston, J.O., Balistreri, W.F., Kramer, L.W., Dresser, B.L. & Tarr, M.J. (1987). Dietary estrogens - a probable cause of infertility and liver disease in captive cheetahs. *Gastroenterology* **93**, 225-233.

Smart, C.C., Johanning, D., Muller, G. & Amrhein, N. (1985). Selective overproduction of 5-enol-pyruvylshikimate acid 3-phosphate synthase in a plant cell culture which tolerates high doses of the herbicide glyphosate. *Journal of Biological Chemistry* **260**, 16338-16346.

USDA Agriculture Handbook No. 8. (1975). Composition of Foods. In Agricultural Handbook No. 8. United States Department of Agriculture, Washington, D.C. pp. 159-165.

Waggle, D.H. & Kolar, C.W. (1979). Types of soy protein products. In: Soy Protein and Human Nutrition. H.L. Wilke, D.T. Hopkins, and D.H. Waggle, eds. Academic Press, New York. pp. 19-51.

Wang, G., Kuan, S.S., Francis, O.J., Ware, G.M. & Carman, A.S. (1990). A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J. Agric. Food. Chem.* **38**, 185-190.

Weiss, U. & Edwards, J.M. (1980). Regulation of the shikimate pathway. *In*: The Biosynthesis of Aromatic Compounds. John Wiley & Sons, New York, pp. 287-301 ●

GM food safety assessment
tools *for* trainers

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ISBN 978-92-5-105978-4

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TC/M/I0110E/1/04.08/2000