

# **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

© World Health Organization 1995

This document is not a formal publication of the World Health Organization (WHO), and all rights are reserved by the Organization. The document may, however, be freely reviewed, abstracted, reproduced and translated, in part or in whole, but not for sale nor for use in conjunction with commercial purposes.

The views expressed in documents by named authors are solely the responsibility of those authors.

Ce document n'est pas une publication officielle de l'Organisation mondiale de la Santé (OMS) et tous les droits y afférents sont réservés par l'Organisation. S'il peut être commenté, résumé, reproduit ou traduit, partiellement ou en totalité, il ne saurait cependant l'être pour la vente ou à des fins commerciales.

Les opinions exprimées dans les documents par des auteurs cités nommément n'engagent que lesdits auteurs.



## Table of Contents

	Page
1. Introduction .. .. .	3
2. Background .. .. .	5
2.1 The concept of substantial equivalence .. .. .	5
2.2 Application of substantial equivalence .. .. .	5
3. Identification of Appropriate Parameters to Establish Substantial Equivalence .. .. .	6
3.1 Molecular characterization .. .. .	7
3.2 Agronomic traits .. .. .	7
3.3 Chemical characterization .. .. .	8
3.3.1 Critical nutrients .. .. .	8
3.3.2 Critical toxicants .. .. .	9
3.3.3 Considerations for both nutrients and toxicants .. .. .	9
4. Identification of the Appropriate Reference Characteristics .. .. .	10
4.1 Analysis of single key components .. .. .	10
4.2 Reference characteristics .. .. .	11
4.3 Validated analytical methods .. .. .	12
4.4 Databases .. .. .	12
5. Conclusions and Recommendations .. .. .	14
Annex 1 List of Participants .. .. .	16
Annex 2 List of Working Papers .. .. .	20
Annex 3 Case study 1:	
♦ Food safety evaluation of transgenic potato by A.J. Conner (New Zealand) .. .. .	23
Annex 4 Case study 2:	
♦ Application of the principles of substantial equivalence in the safety evaluation of FLAVR SAVR tomato, BXN cotton and oil- modified rapeseed by K. Redenbaugh, et al (USA) .. .. .	37
Annex 5 Case study 3:	
♦ Safety evaluation of transgenic tomatoes expressing Bt endotoxin by H.P.J.M. Noteborn and H. Kuiper (The Netherlands) .. .. .	51
Annex 6 Case study 4:	
♦ Safety evaluation of Colorado potato beetle-protected potatoes by Roy Fuchs, et al (USA) .. .. .	63
Annex 7 Glossary .. .. .	79



## 1. INTRODUCTION

A WHO Workshop on the Application of the Principles of Substantial Equivalence to the Safety Evaluation of Foods or Food Components from Plants Derived by Modern Biotechnology was held in Copenhagen, Denmark, from 31 October to 4 November 1994; the list of participants is presented in Annex 1.

The Workshop was opened by Dr Y. Motarjemi, Scientist, Food Safety Unit, WHO. Dr Motarjemi welcomed the participants on behalf of the World Health Organization.

Dr Motarjemi recalled that biotechnology is an area of science and technology which is developing very rapidly, with many potential benefits for the quantity and quality of the food supply. As for any new food technology, the safety of the products derived from this technology needs to be assessed. Therefore, for several years, the World Health Organization has had a programme for developing principles for assessing the safety of foods produced by modern biotechnology. This programme started with the Joint FAO/WHO Consultation on the Assessment of Biotechnology in Food Production and Processing as Related to Food Safety (Geneva, 1990)<sup>1</sup>, and was later followed by a WHO Workshop on Health Aspects of Marker Genes in Genetically Modified Plants (Copenhagen, 1993)<sup>2</sup>.

It was recognized that the work carried out by WHO and other organizations constitutes important milestones in reaching international consensus on principles and procedures for the evaluation of safety of foods produced by modern biotechnology. In this context reference was made to the Codex Alimentarius Commission, which in its medium term programme plans to develop Guidelines for the Evaluation of Foods Produced by Biotechnology. To this end, FAO and WHO plan to jointly convene a Consultation in 1995 to prepare a first draft of the Guidelines. The work which has been carried out by WHO and other organizations, as well as the outcome of the present Workshop will constitute a basis for the development of the above-mentioned Guidelines.

The objective of this specific Workshop is to provide practical guidance as to how the concept of substantial equivalence can be applied as an approach to the safety evaluation of foods or food components from plants derived by modern biotechnology. This is a comparative approach to the assessment of food safety. The comparative approach was first proposed by WHO/FAO, and was further developed by OECD. During a recent OECD Workshop (Oxford, 1994)<sup>3</sup> it

---

<sup>1</sup> *Strategies for Assessing the Safety of Foods Produced by Biotechnology*. Report of a Joint FAO/WHO Consultation, Geneva, World Health Organization, 1991.

<sup>2</sup> *Health aspects of marker genes in genetically modified plants*. Report of a WHO Workshop. Geneva, World Health Organization, 1993 (Document WHO/FNU/FOS/93.6).

<sup>3</sup> OECD Workshop on Food Safety Evaluation, Oxford (U.K.) 12-15 September 1994. Organisation for Economic Co-operation and Development, Paris, France.

was recognized that there are different interpretations in the practical application of substantial equivalence. Dr. Motarjemi hoped that during this present Workshop some of the questions on the practical application of substantial equivalence, raised during the Oxford Workshop, could be answered.

It was emphasized that the Workshop would not address aspects relating to environmental and occupational safety, nor the application of substantial equivalence to animal feeds derived from plants produced by modern biotechnology.

Thanking the participants and representatives of International Organizations (see Annex 1) for their collaboration in this Workshop, Dr Motarjemi expressed WHO's regret that the International Organization of Consumers Unions (IOCU) was unable to be represented in this Workshop. Regrettably, representatives from the International Potato Center (CIP), Peru; the International Rice Research Institute (IRRI), Philippines; the International Maize and Wheat Improvement Centre, Mexico; the Centre for Plant Biotechnology (CINVESTAV), Mexico; and the Chinese Academy of Science were also unable to attend. The unique know-how and experience of these institutions working in developing countries would be missed during this Workshop.

Dr Motarjemi concluded by thanking the National Food Agency of Denmark for hosting, for the second time, a WHO Workshop on food safety issues relating to biotechnology and providing technical and administrative support. She also acknowledged with thanks the contribution of the Nordic Council of Ministers towards the Workshop and facilitating the participation of several participants.

The Workshop elected Dr I. Knudsen as Chairman, Dr O. Fields and Dr M. Toyoda as Vice-chairmen, and Mr P. Mayers as rapporteur for the Workshop. The deliberations of the Workshop were based on a number of working papers (Annex 2) and case studies (Annexes 3-6).

## 2. BACKGROUND

### 2.1 The concept of substantial equivalence

The report of the Joint FAO/WHO consultation, "Strategies for assessing the safety of foods produced by biotechnology", established that the comparison of the final product with one having an acceptable standard of safety provides an important element of safety assessment. The OECD has elaborated this concept and advocated that "the concept of substantial equivalence is the most practical approach to address the safety evaluation of foods or food components derived by modern biotechnology". Substantial equivalence embodies the concept that if a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety. Safety is considered to be a reasonable certainty that no harm will result from intended uses under the anticipated conditions of consumption. This concept of safety embraces both nutritional and toxicological considerations; combined, nutritional and toxicological safety can be described as "wholesomeness".

Potential food safety issues that may arise from plants derived from modern biotechnology can arise either as a result of the intentionally introduced trait/effect, or as a result of unintentional secondary effects (e.g. pleiotropic effects, insertional mutagenesis, or metabolic effects). Substantial equivalence can therefore be assessed by consideration of both the intended and unintended effects of the genetic modification in the food plant.

Similar issues may also arise from plants derived by conventional breeding. However, this report only considers those plants derived using techniques of modern biotechnology, particularly those derived using recombinant DNA techniques.

It is also important to note that although a particular food or food component may not be determined to be substantially equivalent to an existing food or food component, this does not necessarily mean that it is unsafe. Such a food or food component will need to be evaluated on the basis of its composition and properties. However, given the focus of this Workshop, such foods and food components are not considered in this report.

### 2.2 Application of substantial equivalence

Substantial equivalence is a dynamic, analytical exercise which involves a comparison of the relative safety of a new food or food component to an existing food or food component. This comparison may be a simple task or be very lengthy depending upon the extent of experience and the nature of the food or food component under consideration.

A demonstration of substantial equivalence provides reassurance that the new food or food component (e.g. oil, starch, or sugar) is comparable in terms of its safety to its conventional counterpart. It may be possible to establish substantial equivalence for the whole food or food component, or for the food except for the inserted gene and its intended trait(s) or expression products. In the latter situation, the inserted gene and its intended trait or expression product would then be the focus of further evaluation. If the introduced trait can be shown to be substantially equivalent to a trait in a conventional food or food component or demonstrated to present no safety concern, then the whole food will not require further testing to establish safety. However, if the introduced trait cannot be shown to be substantially equivalent to a trait/effect in a conventional food or food component, then further assessment to demonstrate safety is necessary. This assessment would focus on the nutritional and toxicological effects of the introduced trait or expression product and may also involve assessment of the potential for allergenicity where such an effect might be expected from the nature and source of any introduced protein.

In establishing substantial equivalence it is important to remember that the characteristics of food plants will change as a result of a number of factors, including changes in agronomic practices and modifications brought about by traditional breeding as well as modern biotechnology. When a new trait has been introduced into a food plant through modern biotechnology, and the products are deemed to be safe, the new trait becomes part of the point of reference for future developments. Thus a genetically modified food plant, demonstrated to be safe, becomes available as a reference point for the future assessment of substantial equivalence of newly developed varieties.

### 3. IDENTIFICATION OF APPROPRIATE PARAMETERS TO ESTABLISH SUBSTANTIAL EQUIVALENCE

A determination of substantial equivalence can be carried out at the level of the food plant or the specific plant product which will be used as human food. This might entail a consideration of the molecular characterization of the new plant line, agronomic traits and critical nutrients and toxicants (as defined below). It can build on a comparison with the parental line and/or other edible cultivars of the species or it can build on a comparison of the derived plant product (e.g. oil, starch or sugar) with the analogous conventional product. The data required to demonstrate substantial equivalence may come from a variety of sources including existing food component databases, the scientific literature, or field trials carried out on the modified plant with conventional varieties serving as concurrent controls.

### 3.1 Molecular characterization

Molecular characterization, including knowledge of the source and function of the introduced DNA and DNA sequence data, although required in the overall safety assessment, does not, in itself, demonstrate substantial equivalence. However, it is of value in pointing toward relevant parameters which need to be examined. Understanding the inserted genes allows the safety evaluation to focus on the safety of the expression product and/or changes brought about by the expression product. For example, if an amino acid pathway has been altered intentionally, then an assessment of the amino acid profile is necessary.

The inserted DNA and the encoded expression product(s) should be well characterized. Because the DNA molecule itself does not represent any toxicological concern, understanding the level and mechanism of expression of the protein is more important than knowing the gene copy number. Further, the source of the introduced DNA is not of particular concern if the DNA is well characterized and the function of the expression product is known, (i.e. it has been shown that possible toxins or allergens have not been transferred from the donor) but may be of concern if DNA is not well characterized.

Consideration of the level and function of the introduced gene product in the plant may be useful in judging substantial equivalence. Introduced gene products that are similar to substances already present in the plant would not be expected to differ in safety from those substances. For example, an enzyme which is similar or identical to an endogenous enzyme would be expected to be as safe as the endogenous enzyme.

For those gene products for which substantial equivalence cannot be demonstrated, their safety should be assessed on a case by case basis. Parameters that might need to be examined include enzymatic or biological function, digestibility, homology to known toxins, and evaluation of the potential for allergenicity.

### 3.2 Agronomic traits

Because unintended alterations that affect the composition of a plant might be expected to alter agronomic traits, agronomic traits can form a good starting point for evaluating substantial equivalence of genetically modified plants to their conventional counterparts. These traits will normally be examined as an integral part of the development programme leading to a new food plant variety. Agronomic traits are those measured by plant breeders for a given crop (often as dictated by national variety registration requirements and commercial needs). For example, in the case of potatoes these might include yield, tuber size and distribution, dry matter content, disease resistance, and uptake of environmental contaminants.

If deviation from standard crop performance occurs and is undesirable, then those lines will typically be discarded by plant breeders. For those lines carried forward in development, unexpected (unpredicted or unspecific secondary effect) traits may be indicative of unintended effects of potential safety concern and would require further investigation.

### 3.3 Chemical characterization

Chemical characterization for the purpose of assessing substantial equivalence can be divided into three separate but interrelated processes. First, critical components are determined by identifying key nutrients and toxicants (as defined below). Second, data are generated and compared to the parent, other edible varieties of the species and literature values for the species, as appropriate. Finally, the food plant and/or food component(s) is judged either to be substantially equivalent or not substantially equivalent based on these comparisons.

#### 3.3.1 Critical nutrients

Critical nutrients are those components in a particular food product which may have a substantial impact in the overall diet. These may be major constituents (fats, proteins, carbohydrates) or minor compounds (minerals, vitamins). Critical nutrients to be assessed may be determined, in part, by knowledge of the function and expression product of the inserted gene (e.g., if an inserted gene expresses an enzyme which is involved in amino acid biosynthesis, then the amino acid profile should be determined; introduction of an invertase into potatoes could influence the carbohydrate metabolism, therefore starch should be investigated; or, introduction of a storage protein which is high in methionine into soya bean could affect the amino acid profile, therefore the amino acid profile should be determined).

#### 3.3.2 Critical toxicants

Critical toxicants are those toxicologically significant compounds known to be inherently present in the species, i.e. those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased). Critical toxicants to be assessed may be determined, in part, by knowledge of the function and expression product of the inserted gene (e.g., if an invertase is inserted into potatoes, then solanine should be investigated because it is a glycoalkaloid).

It has been suggested that modern biotechnology may inadvertently cause the production of new toxicants through insertional mutagenesis events which activate dormant biosynthetic pathways. While this possibility cannot be absolutely ruled out, it is reasonable to conclude that the greater the experience with traditional breeding of a given crop, the less likely it is that critical toxicants, inherent in the plant but hitherto unexpressed, will be unintentionally induced by genetic modifications carried out using modern biotechnology. It is more likely that the result would be an increase or decrease in the expression of already recognised toxins.

### 3.3.3 Considerations for both nutrients and toxicants

Experiences with genetic modification (case studies presented, Annexes 3-6) support the idea of focusing on the important components of the food plant. Analyzing a broader spectrum of components (i.e. more nutrients and toxicants) is, in general, unnecessary but should be considered if there is an indication from other traits that there may be an unintended effect of the genetic modification.

In addition to analysis of key nutrients and toxicants, the extent of analysis for unintended effects will, in part, be determined by the nature of the intended alteration and by the data from molecular and agronomic characterization. Additional tests may be necessary if these analyses point to possible unintended effects (e.g., a modification leading to the production of a plant hormone will trigger the need to examine those nutrients and toxicants known to be influenced by that hormone).

In addressing the critical nutrients and toxicants in a food plant, analysis of the whole food or food components (raw and/or processed) may be appropriate. For example, soya bean is processed into several products which enter the food supply. If the whole soya bean is demonstrated to be substantially equivalent, it can be extrapolated that the composition of the processed fractions derived from the soya bean are also substantially equivalent. However, in some instances, processed products derived from a genetically modified plant may be substantially equivalent to their conventional counterparts whilst the plant will not be substantially equivalent (i.e. those components that render the whole plant not substantially equivalent to its conventional counterpart might be removed or destroyed during processing).

Given differences among consumption patterns and practices in various cultures and societies, the key nutrients and toxicants to be examined may differ in different regions. Therefore, the critical nutrients and toxicants to be addressed should be determined using consumption data for the target region. The more critical the nutrient or toxicant, the more the attention that must be paid to the implication of comparative differences when establishing substantial equivalence.

#### 4. IDENTIFICATION OF THE APPROPRIATE REFERENCE CHARACTERISTICS

##### 4.1 Analysis of single key components

Establishing substantial equivalence first requires identification of those compounds which need to be compared. Such key compounds (e.g. natural nutrients and toxicants) are described in Section 3. Then, the appropriate reference characteristics (points of reference such as the parental line) need to be determined. This involves taking into account the variation in composition due to genetic variability, environmental factors, and postharvest handling and processing.

A stepwise approach to evaluating the key components is necessary. The first step is to compare the new line to its parent. When there are differences at this level, the new line is compared to other edible varieties of the species. In comparing the new line to the parental line it is important that they be grown under comparable environmental and agronomic conditions. In practice, it may be advantageous to compare the new line with the parent and with other varieties simultaneously due to the length of time required for field growing and the need to control environmental variables.

If the new line is not statistically significantly changed from the parent variety, no further evaluation would be required. Where the genetically modified line is statistically significantly changed from the parent line, comparison to other relevant edible varieties of the species should follow. If this comparison shows that the genetically modified variety is outside the natural range, then additional evaluation of the significance of the differences is required. If the comparison shows that the transgenic variety is within the natural range, then additional evaluation is probably unnecessary.

In either case, determination of what level of variation is acceptable requires taking into account the following issues: 1) whether the change is intended or unintended; 2) whether the concentration of a key compound has been changed; 3) what the intended use of the food is; and 4) what the dietary intake is. For example, an unintended modification in the fatty acid profile of canola would require more evaluation than an intended modification of the fatty acids because it would indicate that an unexpected change to the plant metabolism might have occurred. This could be indicative of a pleiotropic effect that would need further evaluation before such a transgenic crop or product is determined to be substantially equivalent.

#### 4.2 Reference characteristics

From a practical point of view, evaluation of substantial equivalence should typically be performed on the unprocessed food product. For seed crops such as soya bean, canola, cotton and corn, the assessment would focus on the nutrients and toxicants in the unprocessed seed. For tomato and potato varieties, the analytical evaluation would focus on the fresh tomato or tuber, respectively.

However, if the plant resulting from the genetic modification is intended to produce an unprocessed product that is not expected to be substantially equivalent, at the unprocessed product level, to the conventional counterpart, then the substantial equivalence assessment might focus on the appropriate processed food component (e.g. oil from genetically modified rape seed should be compared to traditional rape seed oil or to the oil it is intended to replace in the food supply). The processing applied to food plants and their products that are not substantially equivalent to their conventional counterparts might eliminate components/effects related to the new inserted traits.

The reference characteristics used for comparisons should not be seen as fixed values. They are flexible and can change depending on requirements by breeders, food processors, or consumers. It should also be noted that these reference characteristics may change as experience and knowledge with transgenic crops increase and the safety concerns change. Reference standards for plants derived by conventional plant breeding also vary over time as new varieties are developed (e.g. the levels of erucic acid in edible rape seed oil have been progressively reduced by traditional breeding).

Progeny derived from food varieties shown to be substantially equivalent would be expected themselves to be substantially equivalent. Traditional breeding practices would be expected to reject any varieties in which the inserted trait is unstable or gives rise to adverse secondary effects. This means that a genetically modified potato containing a gene expressing the B.t. toxin could be crossed conventionally with any other potato variety without additional safety evaluation above that normally conducted for conventional new potato varieties.

As transgenic products become more common, it is likely that additional varieties will be developed by crossing varieties, each of which was obtained by genetic engineering. For example, if substantial equivalence has been demonstrated both for a tomato with a gene producing a delayed ripening phenotype and for a tomato with a gene for herbicide resistance, then crossing these two varieties would result in a new variety that would be expected to be substantially equivalent to the parents. As for conventional breeding, potential genetic interactions will need to be considered. For example, if two independent modifications to the same metabolic pathway are combined by traditional breeding, then further analysis of the products of the metabolic pathway may be warranted.

As experience with transgenic crops increases, it is likely that a specific gene will be used in a number of crops and that for each crop substantial equivalence will be demonstrated. This will establish a new reference characteristic, not only for the various crops, but also for the gene product itself. Once this occurs and the gene product has been determined safe in each case, then it will be possible for the gene product to be used in other crops without further testing of the expressed gene product, so long as increased exposure is not a safety concern.

#### 4.3 Validated analytical methods

Analytical assays used to assess the levels of key nutrients and/or toxicants for both conventionally-breed plants as well as genetically modified plants should be standardized assays when available (e.g. methods from the Association of Official Analytical Chemists (AOAC) or other recognized bodies). Methods used to assess the agronomic traits of subject plant varieties should also be standardized methods, if available (e.g. international harmonized guidelines such as Union Pour La Protection D'obtentions Vegetales (UPOV). Standardized methods refer to assays and methods that are validated in terms of accuracy and reproducibility.

#### 4.4 Databases

The relevant data for use as reference standards for traditional-bred plants or genetically modified plants is dependent on the comparisons being made. If the new plant variety (developed by traditional breeding or genetic modification) is being compared directly to the parental variety from which it was derived, then the reference range will be the data developed when the new variety and appropriate parental variety(s) are grown and analyzed side-by-side under a limited number of selected environments that are representative of those environments in which the new variety will be grown commercially. The developer of the new plant variety will typically generate this information.

Comparison of the composition of the new plant variety(s) to other comparable commercial varieties will typically focus on data generated within these varieties grown within similar geographical region(s) in which the new variety is intended to be grown commercially.

Where compositional analysis of key nutrients and toxicants is required for variety registration, data on key nutrients and toxicants will likely be periodically updated and included for current commercial varieties. The published literature on these parameters will also serve as a source for this information. It is important that the reference ranges represent reasonably current information, since the ranges will possibly change over time.

To facilitate appropriate compositional comparisons at the plant species level, it may be useful to use and even generate international databases containing validated data on the nutrients and, especially, toxicant composition of commonly consumed plant varieties. Relevant information could be obtained from the international centers of the Consultative Group on International Agricultural Research (CGIAR). These include the Centers for Genetic Resources Conservation and Breeding Research on specific crops; e.g. CIMMYT (wheat and corn), IRRI (rice), CIP (potato and sweet potato), and CIAT (cassava) and IPGRI (formally IBPGR), which holds the worldwide mandate for the conservation and use of genetic resources. The FAO and the World Food Programme provide other sources of information on food composition.

## 5. CONCLUSIONS AND RECOMMENDATIONS

- ♦ If a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety.
- ♦ Establishment of substantial equivalence is not a traditional safety assessment in itself, but a dynamic, analytical exercise in the assessment of the relative safety of a new food or food component to an existing food or food component. The comparison may be a simple task or be very lengthy depending upon the extent of experience and the nature of the food or food component under consideration.
- ♦ Establishment of substantial equivalence provides sufficient assurance that the foods and food components obtained from a plant derived by modern biotechnology are comparable, in terms of safety, to their conventional counterparts.
- ♦ Substantial equivalence is established for the whole food or food component including the introduced new trait or gene product. In cases where substantial equivalence can be established for the food except for the inserted gene and its trait or gene product, the safety assessment will focus on the latter.
- ♦ Although a particular food or food component is determined not to be substantially equivalent to an existing food or food component this does not mean that it is unsafe. Such a food or food component should be evaluated on the basis of its unique composition and properties.
- ♦ The determination of substantial equivalence entails a consideration of the molecular characterization of the new plant line, its agronomic traits and critical nutrients and toxicants.
- ♦ The identification of appropriate reference characteristics for comparison needs to take into consideration variability caused by genetic factors, environmental factors, postharvest handling and processing, intended use and overall role in the diet at the regional level.
- ♦ The comparison of the new line to traditional food crop plant species should take into account the natural variation and range for parent, variety and species based upon proper statistical analysis. Data for this exercise is derived both in the regional and international setting.

5. CONCLUSIONS AND RECOMMENDATIONS (contd.)

- ♦ In general, progeny derived by traditional breeding from new varieties demonstrated to be substantially equivalent should be assessed on their own merits according to practices for conventional food plants.
- ♦ The reference characteristics for substantial equivalence comparison need to be flexible and will change over time in accordance with changing needs from processors and consumers and with experience gained.
- ♦ WHO is encouraged to consider a future workshop which might consider the possible impact of a wider application of substantial equivalence to the assessment of the safety of novel foods and novel food technologies in a broader sense and on those impacted (i.e. developers, plant breeders, food processors, retailers and consumers).
- ♦ The availability of comparative data on the closest conventional counterpart is critical to the evaluation of foods and food components derived from genetically modified plants and WHO is encouraged to take a leadership role in the development and improvement of international databases on food and food component composition.
- ♦ The principles established for assessing the substantial equivalence of food plants might also be applicable to the food safety assessment of microorganisms and animals derived by modern biotechnology.
- ♦ The principles established for assessing the substantial equivalence of genetically modified food plants and their products might also be useful in the assessment of genetically modified plants which are intended for use as animal feeds and should be brought to the attention of the relevant organizations, such as FAO.

ANNEX 1

**List of Participants**

Dr Christer Andersson	National Food Administration, Uppsala, Sweden
Dr Karl-Heinz Engel	Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany
Dr F. Owen Fields	Biotechnology Policy Branch, Office of Premarket Approval, Centre for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC, USA
Dr Roy Fuchs	Monsanto Company, St. Louis, Missouri, USA
Dr Anja Hallikainen	Senior Scientific Officer, National Food Administration, Helsinki, Finland
Dr Ken-ichi Hayashi	Senior Adviser, Society for Techno-Innovation of Agriculture, Forestry and Fisheries, Tokyo, Japan
Dr Kenji Isshiki	Chief, Food Protection Laboratory, National Food Research Institute, Japan
Dr Sirpa Kärenlampi	Assistant Professor, Department of Biochemistry and Biotechnology, University of Kuopio, Kuopio, Finland
Dr Ib Knudsen	Head, Institute of Toxicology, National Food Agency of Denmark/Ministry of Health, Soborg, Denmark
Dr Paul R. Mayers	Head, Office of Food Biotechnology, Evaluation Division, Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, Ottawa, Canada
Mr Arne Mikalsen	Senior Scientist, Department of Environmental Medicine, National Institute of Public Health, Oslo, Norway
Dr Hub P.J.M. Noteborn	Research Scientist, Department of Risk Assessment and Toxicology, State Institute for Quality Control of Agricultural Products (RIKILT-DLO), Ministry of Agriculture, Nature Management and Fisheries, Wageningen, The Netherlands

Mr Jan Pedersen	Institute of Toxicology, National Food Agency of Denmark/Ministry of Health, Soborg, Denmark
Ms Ranjini Rasaiah	Food Science Division II, Food Safety Directorate, Ministry of Agriculture, Fisheries and Food, London, United Kingdom
Dr Keith Redenbaugh	Manager, Regulatory Affairs, Calgene, Davis, CA, USA
Dr Isabel M.M. Roos	Head of Plant Biotechnology and Pathology, INFRUTEC, Agricultural Research Council, Stellenbosch Institute for Fruit Technology, Stellenbosch, South Africa
Dr Masatake Toyoda	Division of Foods, National Institute of Health Sciences, Ministry of Health and Welfare, Tokyo, Japan
Dr Barend Verachtert	Plant Genetic Systems, N.V., Gent, Belgium
Dr Jocelyn Webster	Division of Food Science and Technology, CSIR, Pretoria, South Africa

**Representatives of Organizations**  
(in alphabetical order)

*Centre for Plant Biotechnology (CINVESTAV), National Polytechnic Institute, Irapuato, Mexico*

[invited but unable to attend]

*Chinese Academy of Sciences, Beijing, People's Republic of China*

[invited but unable to attend]

*European Commission*

Prof. Dr O. Rohte	Directorate General III Industry, Directorate E-1 Foodstuffs-Legislation and Scientific and Technical Aspects, Brussels, Belgium
-------------------	--

*EC Scientific Committee for Food*

Dr Ib Knudsen                      National Food Agency of Denmark

*Food and Agriculture Organization (FAO)*

Dr Victor Villalobos      Senior Officer in Plant Biotechnology, Plant  
Production and Protection Service, Rome,  
Italy

*International Centre for Tropical Agriculture (CIAT), Colombia*

[invited but unable to attend]

*International Food Biotechnology Council (IFBC)*

Dr D. Stephen Saunders    c/o Frito-Lay, Inc., Dallas, Texas, USA  
President, IFBC

*International Life Sciences Institute (ILSI)*

Dr Paulus M. Verschuren    Secretary General/Scientific Director, ILSI  
Europe, Brussels, Belgium

Dr Anthony Huggett        ILSI Europe Novel Food Task Force, Lausanne,  
Switzerland

*International Maize and Wheat Improvement Center, Mexico*

[invited but unable to attend]

*International Organization of Consumers Unions (IOCU)*

[invited but unable to attend]

*International Rice Research Institute, Manila, Philippines*

[invited but unable to attend]

*Joint IUFOST/IUNS<sup>1</sup> Committee on Food, Nutrition and Biotechnology*

Dr Klaus-Dieter Jany      c/o Centre for Molecular Biology, Federal  
Research Centre for Nutrition, Karlsruhe,  
Germany

*Organization for Economic Cooperation and Development (OECD)*

Dr Peter W.E. Kearns      Environment Directorate, Paris, France

Mr Yoshinobu Miyamura      Biotechnology Unit, DSTI, Paris, France

*United Nations Industrial Development Organization, Vienna, Austria*

[invited but unable to attend]

**Secretariat**

Dr Folmer D. Eriksen      Temporary Adviser, Institute of  
Toxicology, National Food Agency of  
Denmark, Soborg, Denmark

Dr David A. Jonas      Temporary Adviser, Biotechnology &  
Novel Foods Branch, Ministry of  
Agriculture, Fisheries and Food,  
London, United Kingdom

Dr Jan Willem van der Kamp      Programme Director Biotechnology TNO,  
Head, WHO Collaborating Centre for the  
Health Impact of Biotechnology, TNO  
Nutrition and Food Research, Zeist, The  
Netherlands

Dr Yasmine Motarjemi      Scientist, Food Safety Unit, Division  
of Food and Nutrition, World Health  
Organization, Geneva, Switzerland

---

<sup>1</sup> International Union of Food Science and Technology/  
International Union of Nutritional Sciences

## ANNEX 2

A number of working papers were presented over the course of the Workshop and served as background for the discussions which led to the development of the report recommendations. The titles of the working papers and author information is listed in this Annex. Some of the working papers may be published in the scientific literature; all enquiries should be directed to the contacts listed below:

Title	Author(s)	Contact
Substantial equivalence, interpretation and application in practice	J. Pedersen, F. Erickson, B.B.L. Jacobsen, I. Knudsen & J. Schlundt	Mr Jan Pedersen Institute of Toxicology, National Food Agency of Denmark, Ministry of Health, Mørkhøj Bygade 19, DK 2860 Søborg, Denmark
New analytical and <i>in vitro</i> techniques for the safety evaluation of novel foods	J. van der Kamp	Dr J.W. van der Kamp, Programme Director, Biotechnology TNO, Director, WHO Collaborating Centre for the Health Impact of Biotechnology, TNO Nutrition & Food Research, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist, The Netherlands
Food safety evaluation of transgenic potato	A.J. Conner	Dr A.J. Conner, Crop Research Division, Department of Scientific and Industrial Research, Private Bag, Christchurch, New Zealand
Application of the principles of substantial equivalence in the safety evaluation of FLAVR SAVR tomato, BXN cotton and oil-modified rapeseed	K. Redenbaugh, J. Lindemann & L. Malyj	Dr Keith Redenbaugh, Manager, Regulatory Affairs, Calgene, 1920 Fifth Street, Davis, CA 95616, USA
Safety evaluation of transgenic tomatoes expressing Bt endotoxin	H.P.J.M. Noteborn and H. Kuiper	Dr H.P.J.M. Noteborn, Research Scientist, Department of Risk Assessment and Toxicology, State Institute for Quality Control of Agricultural Products (RIKILT-DLO), Ministry of Agriculture, Nature Management and Fisheries, Bornsesteeg 45, P.O. Box 230, 6700 AE Wageningen, The Netherlands
Safety evaluation of Colorado Potato Beetle resistant potatoes	R. Fuchs	Dr Roy Fuchs, Monsanto Company, GG4J, 700 Chesterfield Parkway North, St. Louis, Missouri 68198, USA
Delayed softening tomatoes expressing an antisense polygalacturonase gene	F.O. Fields	Dr F. Owen Fields, Biotechnology Policy Branch, Office of Premarket Approval, Centre for Food Safety and Applied Nutrition, Food and Drug Administration, 200 C Street, S.W. Washington, D.C. 20204, USA
UK review of oil from modified rapeseed	R. Razaiah	Ms R. Razaiah, Food Science Division II, Food Safety Directorate, Ministry of Agriculture, Fisheries and Food, Ergon House, c/o Nobel House, 17 Smith Square, London SW1P 3JR, U.K.
Safety assessment of genetically modified plants in Canada: Canola as a case study	P. Mayers	Dr Paul R. Mayers, Head, Office of Food Biotechnology, Evaluation Division, Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, 4th Floor West, Sir Frederick Banting Research Centre, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada
Strategy for the safety evaluation of food produced using biotechnology in Japan	M. Toyoda	Dr Masataka Toyoda, Division of Foods, National Institute of Health Sciences, Ministry of Health and Welfare, 1-81-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan

## INDEX TO CASE STUDIES

Several working papers are presented here as case studies. These reflect examples of products which have been developed using modern biotechnology and examples of the data which the proponents have developed in order to address the parameters identified to use the concept of substantial equivalence as part of the safety assessment. The case studies were prepared by the indicated author(s) and though these were discussed in plenary, there was no attempt to reach consensus on the conclusions they contain. These case studies should not be interpreted as a WHO commentary on the safety of the foods or food components selected.

- |              |   |   |
|--------------|---|---|
| Case study 1 | ♦ | Food safety evaluation of transgenic potato<br>by A.J. Conner (New Zealand)   |
| Case study 2 | ♦ | Application of the principles of substantial<br>equivalence in the safety evaluation of<br>FLAVR SAVR tomato, BXN cotton and oil-<br>modified rapeseed<br>by K. Redenbaugh, et al (USA) |
| Case study 3 | ♦ | Safety evaluation of transgenic tomatoes<br>expressing Bt endotoxin<br>by H.P.J.M. Noteborn and H. Kuiper<br>(The Netherlands)  |
| Case study 4 | ♦ | Safety evaluation of Colorado potato<br>beetle-protected potatoes<br>by Roy Fuchs, et al (USA)  |



CASE STUDY 1

**Food safety evaluation of transgenic potato**

A. J. Conner (New Zealand)



## **CASE STUDY: FOOD SAFETY EVALUATION OF TRANSGENIC POTATO**

A. J. Conner

New Zealand Institute for Crop & Food Research Ltd, Private Bag 4704, Christchurch,  
New Zealand.

### **INTRODUCTION**

One of the crops at the forefront of genetic engineering technology is the cultivated potato (*Solanum tuberosum* L.). This is a result of the importance of the potato crop throughout the world, the relative ease with which the crop can be transformed, and genetic limitations associated with traditional potato breeding (Vayda and Belknap, 1992). Many public research institutes and private companies are targeting potato improvement via the transformation of existing cultivars with specific genes. Consequently, numerous transgenic potato cultivars are anticipated to be commercialised within the next decade.

The first field tests on transgenic potatoes were in the United Kingdom in 1987. Within the next 4 years there were over 70 regulatory approved field trials on transgenic potatoes, excluding multi-site trials of the same material in the same year (Chasseray and Duesing, 1992). These field tests were performed throughout the world and involved evaluation of potato plants transformed with genes conferring a wide range of traits. These included: selectable marker genes and reporter genes, resistance to a wide range of pests, diseases and herbicides, production of thaumatin, and metabolic changes such as altered sugar/starch content and response to bruising (OECD, 1990). Potatoes transgenic for many similar or additional traits are expected to be field tested within the next few years. Some of the transgenic potato lines initially tested in small-scale field experiments are currently being evaluated in scale-up field trials at multiple sites, with the anticipation of commercial releases.

Public concerns about the release and agricultural use of transgenic crop plants have been raised throughout the world. Debates have involved environmental issues relating to the growth of transgenic crops in open fields (Tiedje et al, 1989), and the safety of food produced from these crops (Conner, 1993). In order to gain public acceptance of transgenic crops in the agriculture and food industries it is important that scientifically valid data is collected to provide a rational basis for the assessment of perceived risks.

The aim of this case study is to summarise the issues relating to the food safety evaluation of transgenic potatoes. This involves an overview of relevant aspects of the biology of potato crops, the nature of potato transformation with respect to the integration and expression of transgenes, and the phenotypic appearance of transgenic potato plants. This information is then used as basis to discuss potential issues of concern associated with the assessment of food safety for the commercial use of transgenic potatoes in agriculture. Biosafety data relating to the food safety of transgenic potatoes is summarised to provide initial base-line data on which informed decisions can be made concerning the food safety transgenic potatoes. A more detailed account of the issues and information presented in this case study can be found elsewhere (Conner, 1994; 1995).

## RELEVANT ASPECTS OF POTATO BIOLOGY

The potato (*Solanum tuberosum* L.) is a major food crop throughout the world. It was introduced into Europe from South America in the 16th century and is cultivated for the production of underground tubers, which are storage organs developmentally derived from plant stems (Simmonds, 1976). Potato crops have high productivity in diverse climates and high nutritional value with respect to carbohydrate, protein and vitamin content. They are highly heterozygous and suffer from severe inbreeding depression. Cultivated potatoes are autotetraploids ( $2n = 4x = 48$ ) with specific cultivars being vegetatively propagated as clones.

Wild tuberous *Solanum* species contain high concentrations of potentially toxic glycoalkaloids which are very bitter in taste. The presence of glycoalkaloids in *Solanum* species are generally believed to be a natural plant defense mechanism against pests and diseases. One of the first steps in the domestication of the potato crop is considered to be the emergence of plants with non-bitter tubers (Simmonds, 1976). Modern potato cultivars accumulate high glycoalkaloid concentrations in green shoot tissue and in tubers upon exposure to light. In some cultivars significant concentrations of glycoalkaloids can even accumulate in the tubers not exposed to light. The variation in glycoalkaloid content of tubers can be attributed to both genetic effects and the environmental conditions under which the plants are grown and stored following harvest (van Gelder, 1990). The concentration of glycoalkaloids in potato tubers in advanced lines of modern breeding programmes is usually routinely monitored (Morris and Lee, 1984), and are effectively controlled by statutory or voluntary guidelines. This is important, especially when potato cultivars are developed with germplasm containing wild-species in their ancestry (van Gelder 1990).

## NATURE OF TRANSFORMATION IN POTATOES

Transformation of potatoes is usually achieved by co-cultivating plant tissue with disarmed *Agrobacterium* strains carrying binary vectors. Commonly used potato tissues include leaf segments (eg. De Block, 1988; Wenzler et al, 1989; Conner et al., 1991), minituber segments (eg. Sheerman and Bevan, 1988; Stiekema et al, 1988; Ishida et al, 1989), or anodal stem segments (eg. Visser et al, 1989a; Newell et al, 1991). Following the co-cultivation of plant tissue with *Agrobacterium*, gene transfer only occurs in a small proportion of the potato cells. A method is therefore required to allow the preferential growth and regeneration of rare transformed plant cells. The only effective approach involves the co-introduction of selectable marker genes that confer resistance to phytotoxic chemicals such as antibiotics or herbicides. The neomycin phosphotransferase II gene, originally from the bacterial transposon Tn5, is the most commonly used selectable marker gene for plant transformation. The expression of this gene in plant cells confers resistance to kanamycin and related antibiotics in the cell culture medium, and therefore provides a positive selection for the growth, division and regeneration of transgenic plants. This kanamycin resistance selectable marker system has been routinely used for the recovery of virtually all transgenic potato plants developed to date.

The nature of the DNA targeted for transfer to plant cells by *Agrobacterium* is well characterised. The complete nucleotide sequence of the modified T-DNA on binary vectors is either known or can be deduced from the component DNA fragments. The modified T-DNA maybe incorporated into the potato genome in a complete, truncated or rearranged manner, and may integrate as single copies or tandem repeats at one or more sites (eg. Ooms et al, 1987; Stockhaus et al, 1987; Sheerman and Bevan, 1988; Visser et al, 1989b; Conner et al, 1991). This is consistent with results in other plant species. The amount of DNA integrated is very minute (5-20 kbp), compared to the genome size of commercial potato cultivars (1597-1862 Mbp for a 1C nucleus; Arumuganathan and Earle, 1991). The initially selected transgenic plants regenerated following transformation are hemizygous for inserted transgenes. Due to constraints imposed by the high heterozygosity and inbreeding depression in clonal crops such as potato, these initially selected plants are vegetatively propagated as clones through to commercial release (Conner and Christey, 1994).

Transgene integration is generally recognised as occurring in a "random manner" throughout the genome of plants (Walden et al, 1991). Results from the mapping of the DNA sequences flanking T-DNA insertion sites in potato are consistent with this conclusion (Jacobs et al, 1994). About half the insertion sites involve high-copy/repetitive DNA, and the other half low/single copy DNA (Jacobs et al, 1994). Similarly, the frequency of promoter trapping via T-DNA insertion in potatoes also suggests that approximately half of the T-DNA insertions into the potato genome involve integration into existing genes (Conner et al, 1993). Such integration events raise the possibility of insertional mutagenesis being a relatively common situation during plant transformation.

The magnitude of transgene expression, and sometimes the developmental pattern of expression, varies markedly among populations of independently selected transgenic potato lines (eg. Stiekema et al, 1988; Wenzler et al, 1989; van der Leij et al, 1991). This is consistent with other plants and is generally referred to as the "position effect" (Nap et al, 1993). It is thought to be a consequence of random integration into the plant genome and the influence of the surrounding chromatin on transgene expression. In general, only about 10-20% of transgenic lines usually have the desired magnitude and specificity of transgene expression.

When a line with high expression of the transgene is identified, the continuity and stability of expression can sometimes be affected by the presence of other homologous sequences in the plant genome (Finnegan and McElroy, 1994). This can involve interactions with endogenous plant sequences, when homologous sequences are used in transformation vectors. Alternatively it may involve interactions between multiple copies of transgenes at either allelic or non-allelic loci, which may arise from either re-transformation or sexual crosses between independently derived transformants. These interactions usually involve a loss-of-function of the transgene, with such gene silencing often being triggered by environmental factors, especially when the plants are grown under stress. They may also be a consequence of the integration site of the transgene in the plant genome.

When transgenic potato lines are grown in the field, they are often observed to grow with dramatic phenotypic changes in plant appearance (eg. Dale and McPartlan, 1992; Belknap et al, 1994; Conner et al, 1994). This usually involves changes in shoot and tuber morphology, slow or weak growing plants, and/or reduced tuber yield usually involving a low number of small tubers. These phenotypic changes have been attributed to epigenetic or genetic events occurring during the cell culture and plant regeneration phase of transformation (somaclonal variation). Such phenotypic changes are commonly reported in populations of potato plants regenerated from cell culture (eg. Shepard et al, 1980; Potter and Jones, 1991). The frequency of these off-types among independently derived transgenic potato lines can range from 15-80% depending on the potato cultivar (Jongedijk et al, 1992), and often do not become apparent until plants are grown in the field (Conner et al, 1994). One of the main objectives during initial small-scale field trials involving a new series of transgenic potato lines is the elimination of these off-types.

Potato transformation is therefore highly unpredictable with respect to the integration and expression of transgenes, and the frequency of somaclonal variation among transgenic lines. A large number of independently derived transgenic potato lines must therefore be selected and regenerated for each transformation vector. This is necessary to recover several lines with appropriate expression of the transgene, while maintaining the phenotypic appearance and yield performance of the parental cultivar. In clonal crops, such as potato, this is especially important, since further breeding to eliminate somaclonal variation will result in the loss of the genetic integrity of the parental cultivar (Conner and Christey, 1994).

## **FOOD SAFETY ISSUES RELATING TO THE RELEASE OF TRANSGENIC POTATOES**

The main difficulty associated with the biosafety assessment of transgenic crops is the unpredictable nature of transformation. This unpredictability raises the concern that transgenic plants will behave in an inconsistent manner when grown commercially. However, unpredictable behaviour of plant performance and phenotypic expression of genes recombined into new genetic backgrounds is also commonly observed in traditional plant breeding, especially following wide hybridisations. Although the nature of DNA integration is unpredictable for any specific transgenic plant prior to transformation, the manner in which the modified T-DNA has been integrated, and any resulting position effects on transgene expression, can be accurately determined following transformation. Molecular biology allows the exact components of the modified T-DNA to be controlled in a precise manner with respect to the intended coding regions and their controlling regulatory elements. In contrast, traditional plant breeding commonly involves the transfer of traits from a wild related species. This usually includes large blocks of undefined chromatin linked to and including the gene(s) of interest, and often encodes genes for pest and disease resistance that may be responsible for the accumulation of unknown "toxins". Cultivars bred in this manner have been widely grown in agriculture and accepted in food production for many years. From a biosafety perspective it has been argued that genetic engineering offers a greater confidence for producing the desired outcome compared to traditional genetics and breeding (Conner, 1993).

The emphasis during the biosafety assessment of transgenic potatoes should focus on whether they possess any new risks over those that might be expected from existing potato crops or the release of new potato cultivars resulting from traditional plant breeding. The potential and perceived hazards associated with the food safety of transgenic plants have been thoroughly extensively reviewed (IFBC, 1990). The concerns raised can be attributed to three mechanisms (Conner, 1993): protein products derived from expression of the transgenes, pleiotropic and secondary effects as a consequence of transgene expression, and insertional mutagenesis resulting from transgene integration.

When evaluating the biosafety of transgenic potatoes, the first factor to investigate is whether proteins derived from the expression of the transgene are present in the harvested tubers. Concerns to consider include toxicological, immunological, and allergenic responses. From a positive perspective, the nature of transgene expression products are known, and the specificity and magnitude of transgene expression can be determined. Consequently, the testing and evaluation of any negative concerns is possible. When protein expression products do occur in potato tubers, cooking or processing procedures may eliminate the potential hazard, since many proteins are heat labile.

The expression of transgenes in plants often results in the production of enzymes responsible for catalysing biochemical reactions. Consequently, a range of pleiotropic or secondary effects maybe invoked, including such factors as: product accumulation, substrate depletion, and/or altered metabolic flow-through in downstream biochemical pathways (Conner, 1993). Such effects are dependent on the key regulatory points and rate-limiting steps along biochemical pathways, and may vary between plant species and even between genotypes of the same crop. Nevertheless, potential biochemical outcomes from pleiotropic and secondary effects of transgene expression can be predicted, and appropriate analyses can be performed if possible hazardous situations are envisaged.

The high frequency in which T-DNA insertion occurs into low/single copy DNA or into transcribed regions of the potato genome (see above), raises the possibility that insertional mutagenesis is a relatively common event. Transgene integration may therefore result in the disruption of a native gene at the insertion locus, the effects of which are expected to be inherited in a recessive manner. Inbreeding is necessary to recover homozygous individuals required for the phenotypic expression of such mutational events. Consequently, insertional mutagenic events are not expected among the plants originating as the primary transformants regenerated from tissue culture. Since transgenic lines of clonal crops are propagated vegetatively from the original primary transformant, phenotypic effects resulting from the disruption of native genes are not expected to be observed (Conner and Christey, 1994). This is especially so in potato, which is an autotetraploid and has three other potential alleles to complement an insertional mutagenic event resulting from gene disruption.

In rare instances, insertional mutagenesis may involve gene activation resulting from transcriptional gene fusions ("read-through" from T-DNA promoters), or even the formation of fusion proteins via translational gene fusions. Such events are likely to be expressed in a dominant manner and therefore observed in the primary transformant. With respect to transgenic potatoes, the main concern is the activation of gene(s) responsible for accumulation of glycoalkaloids in tubers. It is therefore important that glycoalkaloid concentrations in transgenic potato tubers are assessed. Such assays are already routinely performed for advanced potato lines in traditional breeding programmes.

Secondary effects of transgene expression and insertional mutagenesis do not present new safety issues with respect to potato production. Identical effects may unwittingly occur during traditional potato breeding programmes, especially those involving wide hybridisations (Conner, 1993). They may arise as a consequence of gene complementation, gene recombination, and chromosomal rearrangements such as translocations and inversions.

## FOOD SAFETY ASSESSMENT DATA ON TRANSGENIC POTATOES

To ensure public acceptance of genetic engineering technology in potato improvement, it is important that food safety assessment data is collected. This provides a scientific basis to substantiate the assumption that transgenic potatoes are no different, other than for the specific trait that is altered, from potatoes arising from traditional methods of breeding and genetic manipulation. As a contribution towards establishing whether transgenic potato plants pose any greater food safety risks than traditional potato plants, we have conducted biosafety evaluations in conjunction with our field testing programme on transgenic potato plants. The most extensive information involves transgenic chlorsulfuron-resistant lines that also express a kanamycin-resistant selectable marker gene and a GUS reporter gene (Conner et al, 1989; Moses et al, 1993).

Determinations of nutritional composition established that the chlorsulfuron-resistant transgenic lines only differed slightly from the parental cultivar in content of dry matter, ash, fat, available carbohydrate, soluble and insoluble dietary fibre, crude protein, and all amino acids (Monro et al, 1993). No nutritional changes were expected to be observed in this study. However, the finding of no change in the content of the branched chained amino acids (valine, leucine and isoleucine) is of interest. The basis for the resistance to chlorsulfuron in the transgenic line involved the expression of a mutant acetolactate synthase gene, which encodes an important step in the pathway toward branched chain amino acids (Haughn et al, 1988). The expression of this transgene is expected to supplement the expression of the endogenous potato gene, resulting in an overexpression of acetolactate synthase in the transgenic line. A suggested secondary effect of overexpressing enzymes that catalyse a step in biochemical pathways is flow through effects to downstream metabolites (Conner, 1993). However such secondary effects are dependent upon the key regulatory points and rate-limiting steps along biochemical pathways, and were not evident with respect to the accumulation of branched chain amino acids in potato transgenic for an additional acetolactate synthase gene.

The samples of potato tubers used for determination of nutritional content were also used in standard rat feeding studies. These trials did not detect any difference between transgenic and control sources of potato tubers with respect to energy digestibility, apparent metabolisable energy, and relative protein value (Monro et al, 1993). However a minor difference was detected in the protein digestibility in one transgenic sample. At the end of a long-term growth trial (55 days) in which potato was the sole source of protein and the major source of energy, rats fed the transgenic line did not differ in their mean weight gain, feed intake or feed conversion efficiency from those fed the parental cultivar (Monro et al, 1993). All rats were observed to be in good health at the end of the trial. Post mortem examination did not reveal any pathological changes or any obvious differences in the superficial appearance of their main organs.

Tubers harvested from the transgenic chlorsulfuron-resistant potato lines were also assessed using the standard cooking tests and sensory evaluations employed in our traditional potato breeding programme. All tuber samples had acceptable scores for sloughing, greying and stem-end blackening following steaming, with no significant differences between any of the transgenic lines and the parental cultivar Iwa (Conner, 1995). Following the preparation of potato crisps, no significant differences were observed in measurements of red light reflectance between the transgenic lines and the parental cultivar, with all lines having acceptable crisp colour (Conner, 1995). Sensory evaluation, using a trained human taste panel selected for their sensory acuity, did not detect any difference between steamed tubers of transgenic and control potatoes (Conner, 1995).

In other quality and sensory evaluations of potato tubers, transgenic for a selectable marker gene conferring kanamycin resistance, we also failed to detect any differences between the parental cultivar and two transgenic lines (Williams et al, 1988; Conner 1995). This involved measurement of specific gravity; assessment of mealiness, sloughing, after-cooking darkening, and taste perception of steamed tubers; and the colour and eating texture of crisps. No change in specific gravity and minimal change on fry colour was observed in 38 transgenic potato lines expressing a kanamycin resistant selectable marker gene plus either a  $\beta$ -glucuronidase gene, an antibacterial gene (cecropin), or an antibruising gene (a tyrosine rich arylphorin) (Belknap et al, 1994). Field trials on the transgenic lines used in these studies showed marked deformities in shoot morphology and poor tuber yield involving a low number of small, malformed tubers during field trials (Belknap et al, 1994; Conner et al, 1994). These changes were attributed to somaclonal variation during the tissue culture phase of transformation (Belknap et al, 1994; Conner et al, 1994). Despite these marked morphological abnormalities, virtually no changes in tuber quality attributes were detected, suggesting that quality traits are relatively stable through transgenic development compared to tuber yield and visual plant phenotype.

## CONCLUSION

The food safety assessment of transgenic potatoes must be considered alongside the existing risks associated with the release of new potato cultivars. It should not be assumed that transgenic potatoes are dangerous and have to be proved to be "safe". Instead biosafety assessment should target whether transgenic potatoes possess any new risks over the generally accepted agricultural and food industry practices associated with existing potato crops, or the genetic manipulation of potatoes via traditional plant breeding.

Published information on the food safety evaluation of transgenic potatoes is limited to lines expressing transgenes conferring kanamycin resistance,  $\beta$ -glucuronidase activity and chlorsulfuron resistance. Nutritional evaluations, rat feeding studies, and cooking and sensory assessments have not detected any substantial differences between these transgenic potato lines and their parental cultivars. This information provides a first step toward the establishment of the food safety of transgenic potatoes, and the use of kanamycin resistance as a selectable marker gene for potato transformation. The data also suggests that quality traits associated with potato tubers are relatively stable through transgenic development compared to tuber yield and visual plant phenotype.

For regulatory approval of transgenic potatoes, the focus of assessment should be on the expression of the introduced transgene. Food safety issues only need to be considered if expression products of this gene accumulate in tubers, and if they are stable through standard cooking practices. New assessment criteria to evaluate indirect effects of gene transfer, such as insertional mutagenesis, and secondary/pleiotropic effects of transgene expression are unnecessary. The trait in potatoes relevant to such effects is the accumulation of glycoalkaloids. This is not a new issue relating to potato improvement, but is routinely monitored as a regular component of traditional breeding programmes.

## LITERATURE CITED

- Arumuganathan, K. and Earle, E.D. 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9: 208-218.
- Belknap, W.R., Corsini, D., Pavsek, J.J., Snyder, G.W., Rockhold, D.R., and Vayda, M.E. 1994. Field performance of transgenic Russet Burbank and Lemhi Russet potatoes. *American Potato Journal* 71: 285-296.
- Chasseray, E. and Duesing, J. 1992. Field trials of transgenic plants - an overview. *Agro-Food Industry Hi-Tech* 3(4): 5-10.
- Conner, A.J. 1993. Food safety issues relating to genetic engineering of crop plants. *Agricultural Science* 6(3): 36-41.
- Conner, A.J. 1994. Analysis of containment and food safety issues associated with the release of transgenic potatoes. In: W.R. Belknap, M.E. Vayda and W.D. Park (Eds), *The Cellular and Molecular Biology of Potatoes, 2nd edition*. CAB International, Wallingford, UK, pp. 245-264.
- Conner, A.J. 1995. Biosafety assessment of transgenic potatoes: environmental monitoring and food safety evaluation. In: D. Jones (Ed.), *Proceedings of the 3rd International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*. USDA/OAB, Washington, D.C., in press.
- Conner, A.J. and Christey, M.C. 1994. Plant breeding and seed marketing options for the introduction of transgenic insect-resistant crops. *Biocontrol Science and Technology*, in press.
- Conner, A.J., Williams, M.K., Abernethy, D.J. and Genet R.A. 1989. Chlorsulfuron-resistant potatoes via *Agrobacterium*-mediated transformation. *Journal of Cellular Biochemistry* 13D: 333.
- Conner, A.J., Williams, M.K., Gardner, R.C., Deroles, S.C., Shaw, M.L. and Lancaster, J.E. 1991. *Agrobacterium*-mediated transformation of New Zealand potato cultivars. *New Zealand Journal of Crop and Horticultural Science* 19: 1-8.
- Conner, A.J., Shum, T.S., Tynan, J.L., Williams, M.K. and Christey, M.C. 1993. Frequency of transcriptional gene fusion during *Agrobacterium*-mediated transformation in potato. *Abstracts of the Third International Symposium on the Molecular Biology of the Potato*. Santa Cruz, California. p. 78.
- Conner, A.J., Williams, M.K., Abernethy, D.J., Fletcher, P.J. and Genet, R.A. 1994. Field testing of transgenic potatoes. *New Zealand Journal of Crop and Horticultural Science* 22: 361-371.
- Dale, P.J. and McPartlan, H.C. 1992. Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings. *Theoretical and Applied Genetics* 84: 585-591.

De Block, M. 1988. Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. *Theoretical and Applied Genetics* 76: 767-774.

Finnegan, J. and McElroy, D. 1994. Transgene inactivation: plants fight back! *Bio/Technology* 12: 883-888.

Haughn, G.W., Smith, J., Mazur, B. and Somerville, C. 1988. Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonyl urea herbicides. *Molecular and General Genetics* 204: 266-271.

IFBC 1990. Biotechnologies and food - assuring the safety of foods produced by genetic manipulation. *Regulatory Toxicology and Pharmacology* 12: S1-S196.

Ishida, B.K., Snyder, G.W. and Belknap, W.R. 1989. The use of in vitro-grown microtuber discs in *Agrobacterium*-mediated transformation of Russet Burbank and Lemhi Russet potatoes. *Plant Cell Reports* 8: 325-328.

Jacobs, J.M.E., te Lintel Hekkert, B., El-Kharbotly, A., Jacobsen, E., Stiekema, W.J. and Pereira, A. 1994. Ac-Ds transposons mapped near disease resistance loci for targeted tagging in potato. In: W.R. Belknap, M.E. Vayda and W.D. Park (Eds), *The Cellular and Molecular Biology of Potatoes, 2nd edition*. CAB International, Wallingford, UK, pp. 21-30.

Jongedijk, E., de Schutter, A.A.J.M., Stolte, T., van den Elzen, P.J.M. and Cornelissen, B.J.C. 1992. Increased resistance to potato virus X and preservation of cultivar properties in transgenic potato under field conditions. *Bio/Technology* 10: 422-429.

Monro, J.A., James, K.A.C. and Conner, A.J. 1993. Comparative nutritional evaluation of a transgenic herbicide-resistant potato and the parent cultivar. *FoodInfo Report No. 6*: 47 pp.

Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of Solanaceae glycoalkaloids, particularly those of potato (*Solanum tuberosum*); a review. *Food Technology in Australia* 36: 118-123.

Moses, T.J., Field R.J. and Conner, A.J. 1993. Field testing of potato lines genetically modified for chlorsulfuron resistance. In: *Proceedings I: 10th Australian Weeds Conference and 14th Asian Pacific Weed Science Society Conference*. Weed Society of Queensland, Brisbane, pp. 319-322.

Nap, J.P., Keizer, P. and Stiekema, W.J. 1992. First-generation transgenic plants and statistics. *Plant Molecular Biology Reporter* 11: 239-249.

Newell, C.A., Rozman, R., Hinchee, M.A., Lawson, E.C., Haley, L., Sanders, P., Kaniowski, W., Tumer, N.E., Horsch, R.B. and Fraley, R.T. 1991. *Agrobacterium*-mediated transformation of *Solanum tuberosum* L. cv. "Russet Burbank". *Plant Cell Reports* 10: 30-34.

OECD 1990. *Database file - field releases of genetically modified organisms*. Organisation for Economic Co-operation and Development, Paris, 94 pp.

- Ooms, G., Burrell, M.M., Karp, A., Bevan, M. and Hille, J. 1987. Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. *Theoretical and Applied Genetics* 73: 744-750.
- Potter, R. and Jones, M.G.K. 1991: An assessment of genetic stability of potato in vitro by molecular and phenotypic analysis. *Plant Science* 76: 239-248.
- Sheerman, S. and Bevan, M.W. 1988. A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors. *Plant Cell Reports* 7: 13-16.
- Shepard, J.F., Bidney, D. and Shahin E. 1980: Potato protoplasts in crop improvement. *Science* 208: 17-24.
- Simmonds, N.W. 1976. Potatoes - *Solanum tuberosum* (Solanaceae). In: N.W. Simmonds (Ed.), *Evolution of Crop Plants*. Longman, London, pp. 279-283.
- Stiekema, W.J., Heidekamp, F., Louwerse, J.D., Verhoeven, H.A. and Dijkhuis, P. 1988. Introduction of foreign genes into potato cultivars Bintje and Désirée using an *Agrobacterium tumefaciens* binary vector. *Plant Cell Reports* 7: 47-50.
- Stockhaus, J., Eckes, P., Blau, A., Schell, J. and Willmitzer, L. 1987. Organ-specific and dosage-dependent expression of a leaf/stem specific gene from potato after tagging and transfer into potato and tobacco plants. *Nucleic Acids Research* 15: 3479-3491.
- Tiedje, J.M., Colwell, R.K., Grossman, R.L., Hodson, R.E., Lenski, R.E., Mack, R.N. and Regal, P.J. 1989. The planned introduction of genetically engineered organisms - ecological considerations and recommendations. *Ecology* 70: 298-315.
- van der Leij, F.R., Visser, R.G.F., Oosterhaven, K., van de Kop, D.A.M., Jacobsen, E. and Feenstra, W.J. 1991. Complementation of the amylose-free starch mutant of potato (*Solanum tuberosum*) by the gene encoding granule-bound starch synthase. *Theoretical and Applied Genetics* 82: 289-295.
- van Gelder, W.W.J. 1990. Steroidal glycoalkaloids: consequences for potato breeding and food safety of utilising wild *Solanum* species in breeding programmes. In: R.F. Keller and A.T. Tu (Eds), *Handbook of Natural Toxins*, Vol. 6. Marcel Dekker Inc., New York.
- Vayda, M.E. and Belknap, W.R. 1992. The emergence of transgenic potatoes as commercial products and tools for basic research. *Transgenic Research* 1: 149-163.
- Visser, R.G.F., Jacobsen, E., Witholt, B. and Feenstra, W.J. 1989a. Efficient transformation of potato (*Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes*. *Theoretical and Applied Genetics* 78: 594-600.
- Visser, R.G.F., Hesseling-Meinders, A., Jacobsen, E., Nijdam, H., Witholt, B. and Feenstra, W.J. 1989b, Expression and inheritance of inserted markers in binary vector carrying *Agrobacterium rhizogenes*-transformed potato (*Solanum tuberosum* L.). *Theoretical and Applied Genetics* 78: 705-714.

Walden, R., Hayashi, H. and Schell, J. 1991. T-DNA as a gene tag. *The Plant Journal* 1: 281-288.

Wenzler, H., Mignery, G., May G. and Park W. 1989. A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. *Plant Science* 63: 79-85.

Williams, M.K., Conner, A.J., Gardner, R.C., Deroles, S.C. and Lammerink, J.P. 1988. Gene transfer to potato using *Agrobacterium*. In: K.S. McWhirter, R.W. Downes and B.J. Read (Eds), *Ninth Australian Plant Breeding Conference, Proceedings*, Agricultural Research Institute, Wagga Wagga, pp. 71-72.



CASE STUDY 2

**Application of the principles of substantial equivalence  
in the safety evaluation of FLAVR SAVR tomato,  
BXN cotton and oil-modified rapeseed**

**K. Redenbaugh, J. Lindemann and L. Malyj (United States)**



Application of the principles of substantial equivalence in the safety  
evaluation of FLAVR SAVR tomato, BXN cotton and oil-modified rapeseed

Keith Redenbaugh, Julianne Lindemann,\* and Lori Malyj

Calgene, Inc., Davis, California, USA

\*Lindemann Consulting, El Cerrito, California, USA

### Definition of Substantial Equivalence

"Substantial equivalence" means the same or essentially the same as a plant or a plant product. "Essentially the same" is defined as statistically the same or within the range naturally found and accepted, as applied to major constituents of a product.

A transgenic plant is substantially equivalent to its originator plant if the important characteristics expected for that species are unchanged. For example, a herbicide resistant cotton is substantially equivalent to cotton because it has all the major characteristics of cotton (form, growth habit, function - fiber, oil and meal production), the modification does not alter the use expected for the plant, and the introduced protein is not a major constituent.

A product derived from a transgenic plant meets the definition of substantial equivalence if it is the same or essentially the same as other plant products in use. For example, oil-free meal derived from high laurate-producing rapeseed is substantially equivalent to meal derived from typical canola-quality rapeseed because it has the same biochemical characteristics (determined statistically). In contrast, the high laurate oil is not substantially equivalent to canola oil or any other vegetable oil because of its unique biochemical characteristics. However, the fatty acid components are GRAS (Generally Recognized As Safe) when evaluated individually because they are present at similar levels in other commonly consumed oils.

### Concepts

There are two ways to determine substantial equivalence of a plant. One is to compare levels of important characteristics and constituents of the plant with the natural ranges that have been published and/or accepted for that species of crop. Published natural ranges are often mean values and as such tend to underestimate the actual extent of variation (IFBC 1990). The other way is to demonstrate that the characteristics are statistically equivalent. This is important because levels of certain plant constituents may not be known or may be incompletely described in the literature, but they can be measured in commercial varieties for comparison with the transgenic plant at the time the latter is being characterized.

In general, to be considered substantially equivalent, transgenic plants should be unchanged except for the intended effect, and any new substances should be minor constituents. Judgment is required to determine which characteristics need to be measured and which are unimportant. Principle issues are important nutrients, natural toxicants, and the normal use of the plant.

Using Calgene's products as examples, the intended effect may be delayed ripening in tomato, herbicide resistance in cotton, or modified oil characteristics in rapeseed. Such modifications will determine whether the plant or plant product is substantially equivalent, given that there are no unintended changes. These three examples, which represent Calgene's direct experience, illustrate the principles of substantial equivalence.

### FLAVR SAVR™ Tomato Example

FLAVR SAVR™ tomato is *Lycopersicon esculentum* which contains an antisense polygalacturonase gene that slows fruit softening, allowing the tomatoes to remain on the vine longer thereby improving taste. Examples of substantial equivalency in tomato are:

- 1) **Nutrients:** There was no variation in the major vitamins, protein amount and minerals as compared with non-transgenic tomato controls and natural ranges (Table 1).

**Table 1. Ranges of Nutrients in FLAVR SAVR™ tomato.**

Constituent	Normal range	Transgenics	Controls
Protein	0.85 g (.015 se)	0.75-1.14	0.53-1.05
Vitamin A	192-1667 IU	330-1600	420-2200
Thiamin	16-80 µg	38-72	39-64
Riboflavin	20-78 µg	24-36	24-36
Vitamin B <sub>6</sub>	50-150 µg	86-150	10-140
Vitamin C	8.4-59 mg	15.3-29.2	12.3-29.2
Niacin	0.3-0.85 mg	0.43-0.70	0.43-0.76
Calcium	4.0-21 mg	9-13	10-12
Magnesium	5.2-20.4 mg	7-12	9-13
Phosphorus	7.7-53 mg	25-37	29-38
Sodium	1.2-32.7 mg	2-5	2-3

- 2) **Glycoalkaloids:** There were no differences in tomatine amounts between FLAVR SAVR tomatoes and non-transgenic tomatoes for either green or ripe fruit (Table 2). These levels were within the normal range of tomatine in tomato fruit. Also, as in other tomato varieties, there was no detectable solanine in ripe fruit.

**Table 2. Tomatine levels**

Fruit Stage	Transgenics	Controls
Green	0 - 8.79 mg/100 g fwt	0 - 6.48
Red	0 - 1.09 <sup>a</sup>	0 - 2.31 <sup>b</sup>

<sup>a</sup>Only 1 of 38 fruit had detectable tomatine

<sup>b</sup>Only 4 of 60 had detectable tomatine

The U.S. Food and Drug Administration completed its evaluation of the FLAVR SAVR tomato, concluding that it was substantially equivalent to other tomatoes:

- "FLAVR SAVR™ tomatoes have not been significantly altered when compared to varieties of tomatoes with a history of safe use" (Fed. Reg. 1984).
- The FLAVR SAVR tomato "is as safe as tomatoes bred by conventional means" (FDA, May 17, 1994).

### BXN™ Cotton Example

BXN™ cotton is *Gossypium hirsutum* which contains a nitrilase gene that makes the cotton plant resistant to the herbicide bromoxynil. Substantial equivalency with traditionally-developed cotton varieties was demonstrated by compositional analyses of major constituents. Examples of analyses are:

- 1) Nutrients: Fatty acid composition of cottonseed oil from BXN cotton was comparable to the parental variety and was within the Codex Standard 22-1981 for edible cottonseed oil (Table 3). Amino acid composition was comparable for cottonseed meal from BXN cotton with meal from commercial varieties and within the range of variability for the analytical technique (Table 4).

Table 3. Cottonseed Oil Composition Data (% wt/wt)

CONTROLS			BXN COTTON											
	CODEx STAN <sup>1</sup>	CS Oil <sup>2</sup>	1 C315 <sup>3</sup>	2 C315 <sup>3</sup>	3 C315 <sup>3</sup>	4 10103	5 10109	6 10206	7 10208	8 10209	9 10211	10 10215	11 10222	12 10224
C<14	<0.1	0.07	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.05	0.04	0.03	0.03
C 14:0	0.4-2.0	0.90	0.70	0.88	0.89	0.70	0.76	0.72	0.67	0.64	0.69	0.68	0.72	0.70
C 16:0	17.0-31.0	22.53	25.68	26.26	26.36	24.05	26.10	24.67	24.49	24.28	24.50	24.93	24.65	24.54
C 16:1	0.5-2.0	0.63	0.52	0.56	0.58	0.47	0.53	0.48	0.46	0.45	0.46	0.51	0.47	0.48
C 18:0	1.0-4.0	2.62	2.82	2.64	2.69	2.76	2.63	2.64	2.80	2.97	2.78	2.67	2.83	2.60
C 18:1	13.0-44.0	19.65	15.51	15.58	15.79	15.24	14.21	14.40	15.03	14.63	14.28	14.84	13.72	14.31
C 18:2	33.0-59.0	52.37	53.87	53.05	52.65	55.83	54.68	56.12	55.73	56.11	56.30	55.49	56.72	56.48
C 18:3	0.1-2.1	0.43	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.19	0.17	0.18	0.18
C 20:0	<0.7	0.33	0.31	0.34	0.35	0.31	0.36	0.33	0.31	0.31	0.33	0.30	0.30	0.31
C 20:1	<0.5	0.11	0.07	0.08	0.08	0.08	0.09	0.08	0.08	0.09	0.09	0.08	0.08	0.09
C 22:0	<0.5	0.21	0.16	0.20	0.20	0.18	0.20	0.18	0.17	0.21	0.21	0.17	0.17	0.19
C 22:1	<0.5	0.03	0.04	0.03	0.03	0.03	0.05	0.03	0.01	0.03	0.03	0.03	0.04	0.02
C 24:0	<0.5	0.07	0.11	0.13	0.14	0.12	0.13	0.13	0.00	0.00	0.00	0.00	0.00	0.00

<sup>1</sup> Codex Stan 22-1981 for edible cottonseed oil (Smith 1990)

<sup>2</sup> Example of commercial cottonseed oil, product House of Tsang Wok Oil - also contains natural flavors of garlic, onion, ginger, coriander and black pepper.

<sup>3</sup> Control cotton lines.

<sup>4</sup> Essential fatty acid linoleic acid.

Table 4. Summary of Amino Acid Analysis of Cottonseed Meal<sup>a</sup>

Sample #	Cysteine	Proline	Aspartic Acid	Serine	Threonine	Glutamic Acid	Glycine	Alanine	Valine
1 C315 <sup>b</sup>	1.7	3.6	10.0	5.2	3.9	20.8	4.3	4.3	5.9
2 C315 <sup>b</sup>	1.7	3.7	10.1	4.9	3.6	21.8	3.9	3.9	5.3
3 C315 <sup>b</sup>	1.7	3.7	10.1	5.1	3.4	21.8	3.9	3.8	5.5
4 10103	1.8	3.7	10.0	4.9	3.6	21.7	3.9	3.9	5.2
5 10109	1.8	3.8	10.2	4.7	3.5	21.8	3.9	3.9	5.1
6 10206	1.7	3.7	10.2	5.0	3.5	21.7	3.8	3.7	5.2
7 10208	1.7	3.7	10.1	5.1	3.7	21.5	3.9	3.8	5.4
8 10209	1.8	3.7	10.0	5.2	3.7	21.7	3.9	3.8	5.3
9 10211	1.7	3.7	10.5	5.0	3.5	21.5	3.9	3.8	5.2
10 10215	1.8	3.7	10.2	4.9	3.6	21.7	4.0	3.9	5.5
11 10222	1.8	3.7	10.3	5.0	3.5	21.8	3.9	3.9	5.2
12 10224	1.8	3.6	10.5	5.2	3.6	21.8	3.9	3.9	5.3
Solvent <sup>c</sup>	-	-	-	-	3.4	-	5.1	-	6.2
Prepressed solvent <sup>c</sup>	-	-	-	-	3.2	-	4.1	-	4.5
Mechanical <sup>c</sup>	-	-	-	-	3.2	-	5.0	-	4.7

Sample #	Methionine	Isoleucine	Leucine	Tyrosine	Phenyl alanine	Histi- dine	Lysine	Arginine
1 C315 <sup>b</sup>	1.6	3.6	6.6	3.2	5.6	3.0	5.5	11.2
2 C315 <sup>b</sup>	1.5	3.5	6.3	3.1	6.0	3.0	4.9	13.0
3 C315 <sup>b</sup>	1.5	3.6	6.3	3.0	5.9	3.0	4.9	12.8
4 10103	1.7	3.6	6.3	3.3	5.8	3.0	5.1	12.5
5 10109	1.6	3.5	6.3	3.0	5.8	3.0	5.0	13.1
6 10206	1.5	3.5	6.2	3.1	5.8	3.1	4.9	13.4
7 10208	1.6	3.7	6.2	3.1	5.8	3.0	5.0	12.8
8 10209	1.5	3.6	6.2	3.2	5.9	3.0	4.9	12.8
9 10211	1.3	3.6	6.2	3.3	5.8	3.0	4.8	13.3
10 10215	1.5	3.5	6.3	3.3	5.7	3.0	4.8	12.7
11 10222	1.5	3.4	6.2	3.6	5.7	3.0	4.9	12.8
12 10224	1.4	3.5	6.2	3.0	5.6	3.0	4.9	12.8
Solvent <sup>c</sup>	1.5	3.8	6	-	5.2	2.7	4.1	10.3
Prepressed solvent <sup>c</sup>	1.3	3.2	-	-	5.4	2.6	4.1	11.1
Mechanical <sup>c</sup>	1.4	3.7	5.6	-	5	2.5	3.8	9.9

<sup>a</sup> % wt amino acid in cottonseed meal protein.

<sup>b</sup> Control cotton line.

<sup>c</sup> Data from Ensminger et al. 1990 (Table 11.5)

- 2) **Toxicants:** Gossypol and cyclopropenoid fatty acids are naturally present in cotton and are considered to be anti-nutritional compounds undesirable for food and feed safety. Statistical analyses confirmed that gossypol and CPFA levels in BXN cotton lines were not elevated from the parent variety and were within the range expected for other cotton varieties (Tables 5 and 6).

**Table 5. Comparison of Means for Free Gossypol Levels (% of seed weight)**

Strain <sup>1</sup>	Overall		MS		SC		AZ	
	Mean		Mean		Mean		Mean	
Stv453	1.06	a <sup>2</sup>	1.10	a	0.90	ab	1.20	a
LA887	1.03	ab	0.97	b	0.96	a	1.17	a
BXN10211-20	0.96	abc	0.92	bc	0.87	bc	1.08	b
BXN10211-1	0.93	bcd	0.90	bc	0.86	bc	1.02	bcd
C315	0.90	cd	0.89	bc	0.79	cde	1.04	bc
BXN10222-1	0.89	cd	0.88	bc	0.79	cde	1.02	bcd
BXN10217-15	0.86	cd	0.81	cd	0.76	de	1.00	cde
DPL5415	0.83	d	0.73	d	0.82	bcd	0.95	de
BXN10217-1	0.83	d	0.80	cd	0.73	e	0.94	e
LSD 0.05	0.10		0.12		0.08		0.07	
CV	11.9		9.5		7.0		4.8	

<sup>1</sup> Samples with the BXN prefix are BXN cotton; C315, DPL 5415, LA887, and Stoneville 453 are current commercial varieties of cotton.

<sup>2</sup> Strains within a location containing the same letter are not significantly different at a 95% confidence level.

Table 6. Statistical Analysis of CPFA levels in cottonseed from 3 locations.

Strain <sup>1</sup>	Overall CPFA% <sup>2</sup>	MS CPFA%	SC CPFA%	AZ CPFA%
BXN10217-1	0.67 a <sup>3</sup>	0.62 a	0.69 abc	0.74 * <sup>4</sup>
DP 5415	0.68 ab	0.66 ab	0.56 a	0.86
BXN10222-1	0.71 abc	0.71 abc	0.59 a	0.78
BXN10217-15	0.71 abc	0.67 ab	0.57 a	0.76
BXN10211-20	0.72 abc	0.74 bcd	0.60 a	0.78
C315	0.72 abc	0.67 ab	0.70 abc	0.73
BXN10211-1	0.73 abc	0.73 bcd	0.63 ab	0.82
Stv453	0.76 bc	0.81 cd	0.75 bc	0.80
LA887	0.79 c	0.82 d	0.80 c	0.88
	LSD= 0.08 CV= 19.50%	LSD= 0.1 CV= 17.95%	LSD= 0.14 CV= 20.62%	LSD= NS CV= 15.80%

<sup>1</sup> Samples with the BXN prefix are BXN cotton; C315, DPL 5415, LA887, and Stoneville 453 are current commercial varieties of cotton.

<sup>2</sup> Percentage of CPFA in oil extracted from whole cottonseed, based upon calculation from the *Sterculia* standard.

<sup>3</sup> Strains within a location containing the same letter are not significantly different at a 95% confidence level.

<sup>4</sup> At the Arizona location there were no significant differences among strains.

### Laurate Canola Example

Laurate canola is *Brassica napus* (rapeseed) which produces high levels of lauric acid in its oil, unlike commercial canola varieties which contain no detectable lauric acid. Further, the overall fatty acid composition and triglyceride structures in Laurate canola are not identical to those in lauric oils derived from coconut and palm kernel oils. Thus, oil from laurate canola is not substantially equivalent to any other food oils, even though it shares many of the same characteristics. However, the fatty acid components are GRAS when evaluated individually because they are present at similar levels in other commonly consumed oils.

- 1) **Fatty acid profile:** Laurate canola contains lower levels of oleic acid (18:1) and elevated levels of laurate (C12:0) and myristate (C14:0) compared to Codex specifications for canola oil (Table 7). Levels of other fatty acids in Laurate canola are within the Codex specification. Importantly, levels of the naturally occurring toxicant, erucic acid, are very low in Laurate canola (Table 8). Substitution of Laurate canola for coconut and palm kernel oils does not raise any safety concerns for the intended uses, in part because the major components, the fatty acids laurate and myristate, are identical. Other reasons that Laurate canola does not raise safety concerns, in spite of its lack of substantial equivalence, are beyond the scope of this paper.

**Table 7.** Fatty acid profile of Laurate canola and commercial canola varieties. Values within columns followed by the letter "a" are outside of the Food Chemicals Codex specification for canola oil.

Variety	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
A123L	ND	ND	0.1	3.90	0.3	1.80	61.12	22.25
A112	ND	ND	0.1	3.8	0.3	2.25	66.2	17.4
Cyclone	ND	ND	0.1	4.0	0.3	1.70	61.25	19.6
212/86	ND	ND	0.1	4.5	0.3	1.80	59.3	19.2
Delta	ND	ND	0.1	4.4	0.3	2.0	61.6	19.6
Iris	ND	ND	0.1	4.0	0.3	1.70	59.95	21.6
Laurate	0.1	39.75 a	4.15 a	2.8	0.2	1.35	32.55 a	12.27
Legend	ND	ND	0.1	3.8	0.3	2.2	63.65	18.5
Westar	ND	ND	0.1	3.65	0.3	1.8	63.2	17.8

Variety	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1	C24:0	C24:1
A123L	7.30	0.60	1.30	0.10	0.30	0.05	0.20	0.20
A112	7.80	0.40	0.80	0.10	0.20	ND	0.10	0.10
Cyclone	10.10	0.60	1.30	0.10	0.30	ND	0.20	0.20
212/86	11.05	0.60	1.25	0.10	0.30	0.4	0.20	0.10
Delta	8.80	0.70	1.20	0.10	0.30	0.1	0.20	0.20
Iris	9.30	0.60	1.20	0.10	0.30	ND	0.20	0.20
Laurate	7.37	0.45	0.75	0.05	0.25	ND	0.10	0.10
Legend	8.10	0.75	1.30	0.10	0.40	0.1	0.20	0.20
Westar	9.40	0.65	1.60	0.10	0.40	0.2	0.20	0.20

**Table 8.** Ranges of erucic acid levels in oil from commercial varieties of *B. napus* canola (standards) and Laurate canola compared to literature values and the canola oil specification.

	Specification	Literature values	Standards	Laurate Canola
% by weight of oil				
Erucic acid (C22:1)	≤ 2	0.1 - 0.5	< 0.1 - 0.4	< 0.1

In contrast to the oil, meal prepared from Laurate canola is substantially equivalent to meal from other canola varieties because it is unchanged in important components. Significance can be gauged from regulations and guidelines of expert bodies (e.g. AAFCO, Association of American Feed Control Officials). For example:

- 1) **Nutrients:** Levels of significant nutrients were within the expected range of variation of the standards i.e. were significantly equivalent. For example, amino acids were comparable (Table 9).

Table 9. Comparison of absolute levels of essential amino acids in canola meals (weight % in meal)

Amino acid	Literature values <sup>1</sup>	9 standard varieties <sup>2</sup>	Laurate canola <sup>3</sup>
Arginine	2.1 - 2.79	2.23 - 3.19	2.67 - 2.72
Histidine	1 - 1.19	1 - 1.42	1.17 - 1.2
Isoleucine	1.28 - 1.59	1.56 - 2.1	1.72 - 1.75
Leucine	2.5 - 2.93	2.56 - 3.36	2.88 - 2.93
Lysine	2.1 - 2.32	2.21 - 2.82	2.41 - 2.49
Methionine	0.68 - 0.76	0.78 - 1.18	0.97 - 1.06
Phenylalanine	1.4 - 1.79	1.39 - 1.96	1.64 - 1.72
Threonine	1.6 - 1.91	1.67 - 2.1	1.77 - 1.83
Tryptophan	0.4 - 0.52	0.37 - 0.58	0.39 - 0.46
Valine	1.8 - 1.94	1.96 - 2.62	2.24 - 2.25

<sup>1</sup>Bell 1989, Clandinin 1989, National Research Council 1982 (ranges of mean values).

<sup>2</sup>Ranges from 19 total samples

<sup>3</sup>Range of 3 samples from pooled seed meal

- 2) Toxicants: The level of glucosinolates in Laurate canola meal was less than 30  $\mu\text{mol}$  of any mixture of but-3-enyl, pent-4-enyl, 2-hydroxybut-3-enyl and 2-hydroxypent-4-enyl glucosinolate per gram of air dry, oil free solid, as per AAFCO requirements (Table 10). Levels of individual glucosinolates in Laurate canola meal were not significantly different from the range of those measured in 9 standard varieties.

Table 10. Levels of regulated glucosinolates in the AAFCO specification, in representative commercial varieties, and in Laurate canola compared to levels reported in the scientific literature.

Glucosinolate	Specification	Literature values	Standards	Laurate Canola
$\mu\text{mol/g}$ defatted meal				
Total	< 30		3.7 - 26.5	9.9 - 15.3
3-butenyl		2.08 - 4.3	1.0 - 7.7	3 - 5.7
4-pentenyl		0.53 - 0.7	ND- 2.6	1.4 - 2.9
2-hydroxy-3-butenyl		4.32 - 8.9	2.7 - 15.8	5.3 - 6.4
2-hydroxy-4-pentenyl		0.47 - 0.5	ND- 1.1	0.2 - 0.3

ND = none detected.

- 3) Protein The level of crude protein in Laurate canola meal met AAFCO requirements to exceed 35% (Table 11) and was not significantly different from the range of protein measured in 9 standard varieties.

Table 11. Mean levels of crude protein in meal prepared from 9 commercial canola varieties and Laurate canola.

Variety	% Crude Protein
A123L	44.04
A110-L	46.27
A112	41.31
Cyclone	36.92
212/86	35.55
Delta	41.39
Iris	38.78
Laurate	41.23
Legend	42.83
Westar	44.42

#### Further Analyses

In addition to demonstrating substantial equivalency for a genetically engineered crop or plant product derived from such a crop, molecular and genetic analyses can be conducted to provide characterization beyond that normally done for a new plant variety or improved plant product. Examples include:

- 1) Genetic construction characterization includes preparation of a full DNA sequence map, open reading frame analysis (to demonstrate a lack of DNA sequences that could code for an allergen or toxicant), comparison of the inserted sequences with known toxicants and allergens (to demonstrate lack of significant homology). Construct characterization analysis is done once for each construct.
- 2) Transformation event characterization includes a screen for presence of DNA beyond the T-DNA borders (if necessary) and multi-generational Southern blots (to demonstrate genetic stability and number of insertion loci). This analysis is done once for each transformation event.

## Conclusion

These recommendations are suggested for the first products developed using genetic engineering (Table 12). As experience is gained and data accumulate, in particular for a specific crop, then some of the data recommendations may no longer be necessary for safety assessment. It should be possible to make broad determinations of safety for classes of products. For example, after the first few altered fruit ripening tomato, herbicide resistant cotton, or oil-modified rapeseed varieties are determined to be safe for environmental release, it will be appropriate to issue a broad determination for all examples of the same type in the same crops. Likewise, it is expected that after human and animal safety issues are resolved for the initial varieties, then specific quality (safety) assurance tests will be sufficient and appropriate to assure health and safety. Demonstration of substantial equivalency of the initial products will provide considerable support for such a safety assessment approach and for deregulation of genetically engineered crop varieties. In cases where substantial equivalency cannot be demonstrated, other criteria will need to be used for a determination of safety, such as those articulated by FDA (1992) or IFBC (1990).

Table 12. Status of Near-Term Products as of 11/1/94

Category	Crop	Gene	FDA Consultation completed	Other Status	Company
Delayed ripening fruit	Tomato*	Flavr Savr™ (ASPG)	5/18/94	1, 2, 3, 6 & 7 in review	Calgene
Delayed ripening fruit	Tomato	ACC deaminase	9/19/94	2	Monsanto
Delayed ripening fruit	Tomato	ASPG	9/20/94	2, 8?	Zeneca
Delayed ripening fruit	Tomato	ACC synthase	10/4/94	2, 3 in review	DNAP
Insect resistance	Potato	B.t.t.	9/23/94	4, 5 in review	Monsanto
Virus resistance	Squash	Coat protein	10/3/94	4, 3 in review	Asgrow
Herbicide resistance	Cotton	BXN™ gene (nitrilase)	9/21/94	2, 3	Calgene
Herbicide resistance	Soybean	EPSPS	9/19/94	3, 8?	Monsanto
Herbicide resistance	Flax	EPSPS		6 in review	Univ. of Sask.
Herbicide resistance	Corn	Basta tolerant			AgrEvo
Herbicide resistance	Rapeseed	Basta tolerant		2, 6	AgrEvo
Oil modification	Rapeseed	Thioesterase	in progress	2, 3	Calgene
Sterility	Rapeseed	Bar		2, 8, 6 in review	PGS
Insect resistance	Cotton	B.t.k.		2, 4, 5 in review	Monsanto
Insect resistance	Corn	B.t.k.		4, 5 in review	Ciba-Geigy
Virus resistance	Tobacco*	Coat protein		not known	China

\*Commercialized

<sup>1</sup>Formal safety review by FDA

<sup>2</sup>FDA rule that APH(3')II is safe as a processing aid

<sup>3</sup>USDA "Determination of Non-regulated Status"

<sup>4</sup>EPA exemption from tolerance for NPTII (APH(3')II) as pesticide inert

<sup>5</sup>EPA registration and review of B.t.

<sup>6</sup>Health Canada "No Objection to Sale"

<sup>7</sup>Agriculture Canada deregulation

<sup>8</sup>MAFF UK approval to sell novel food products

## References

- Association of American Feed Control Officials (AAFCO). 1994. Official Publication. College Station TX.
- Bell, J. M. 1989. Nutritional characteristics and protein uses of oilseed meals. Pages 192 - 207 In Robbelen, G., R. K. Downey and A. Ashri (Eds.) *Oil Crops of the World*. McGraw-Hill. New York.
- Clandinin, D. R. 1989. Canola Meal for Livestock and Poultry. Canola Council of Canada, Winnipeg, Manitoba, September 1989.
- Committee on Food Chemicals Codex. 1992. *Food Chemicals Codex*. Third Supplement to the Third, ed. Washington, D.C., National Academy Press.
- Ensminger, M.E., J.E. Oldfield and W.W. Heinemann. 1990. Excerpts with reference to cottonseed and cottonseed components. In: *Feeds and Nutrition*. (M.E. Ensminger, ed.). Clovis, California, Ensminger Publishing Company.
- FDA. 1992. Statement of Policy: Foods derived from new plant varieties. Fed. Reg. 57:22983-23005. May 29, 1992.
- FDA. May 17, 1994. Letter to Calgene on FLAVR SAVR tomato. (FMF 526 and Docket No. 91A-0330).
- Fed. Reg. 59:26647-26648, 1994. Calgene, Inc.; Availability of Letter Concluding Consultation (Docket No. 91A-0330).
- Hilditch, T.P. and Williams, P.N. 1964. The chemical constitution of natural fats. John Wiley & Sons. New York.
- International Food Biotechnology Council (IFBC). 1990. Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regul. Toxicol. Pharmacol. 12(3) Part 2: S11-S78.
- National Research Council. 1982. *United States-Canadian Tables of Feed Composition*. Third, ed. Washington, D.C., National Academy Press.
- Smith, B. L., ed. 1990. *Codex Alimentarius Abridged Version*. Joint FAO/WHO Food Standards Programme. Codex Alimentarius Commission. Rome, Italy, Food and Agriculture Organization of the United Nations and World Health Organization. 11.6 pages.
- Souci, S.W., Fachmann, W., and Kraut, H. 1989. Food Composition and Nutrition Tables 1989/90. Wissenschaftliche Verlagsgesellschaft, Stuttgart.



CASE STUDY 3

**Safety Evaluation of Transgenic Tomatoes  
expressing *Bt* Endotoxin**

Hubert P.J.M. Noteborn and Harry A. Kuiper

State Institute for Quality Control of Agricultural Products (RIKILT-DLO),  
Department of Risk Assessment and Toxicology, P.O. Box 230, 6700 AE Wageningen, The Netherlands



## SAFETY EVALUATION OF TRANSGENIC TOMATOES EXPRESSING *Bt* ENDOTOXIN

Hubert P.J.M. Noteborn and Harry A. Kuiper

State Institute for Quality Control of Agricultural Products (RIKILT-DLO),  
Department of Risk Assessment and Toxicology, P.O. Box 230, 6700 AE Wageningen, The Netherlands

### SUMMARY

Transgenic *Bt*-toxin tomatoes were engineered which contain a gene of *Bacillus thuringiensis* (*Bt*) encoding the CRYIA(b) protein, and a selectable marker gene encoding the enzyme neomycin phosphotransferase II (NPTII). Animal studies and *in vitro* experiments in tissues from rodents, Rhesus monkeys and humans indicated the absence of specific binding sites for CRYIA(b) and of acute pathologic effects. Short-term toxicity testing with CRYIA(b) revealed no adverse effects in mice and rabbits, and no evidence was found for immunotoxicity of the protein. Chemical analysis did not show differences in the nutritional composition of transgenic *Bt*-toxin tomatoes compared to that of parental lines. Moreover, levels of the glycoalkaloid  $\alpha$ -tomatine were similar. Analysis of the results of a 90 day feeding trial of transgenic *Bt*-toxin tomatoes in rats, did not reveal any signs of adverse effects.

### INTRODUCTION

Application of genetic transformation of plants broadened the range of genes that can be inserted into existing crops, as recombinant DNA techniques can bridge the barriers between widely different species. Traits like for insect and virus resistance, herbicide tolerance and delayed ripening of fruits have been introduced in various food plants (1-6). Many aspects of genetic modification by means of novel biotechnology are not new, and to a great extent, already experienced before. In conventional plant breeding selection might have guarded safety, although it was not recognised as such. Nevertheless, recDNA techniques enable breeders to express proteins in plants that were never part of it before. As a consequence, the safety and nutritional value of the novel food is a topic of concern for the consumer. Considerations, which have led to national and international consultations on the necessity of additional regulation for genetically modified plants and derived foodstuffs. As yet, the food safety testing of transgenic food plants is still in a phase of exploration.

In an international context criteria for safety evaluation of novel foods are far from settled. In view of proposed regulations, it can be anticipated that test strategies will show major differences. Growing agreement exists on considering the newly expressed proteins as additives of which the toxicity and exposure levels should be estimated. Whereas possible secondary effects (i.e. pleiotropic effects, insertional mutagenesis, metabolic effects) in transgenic plants, as a result of the genetic modification, should be also analysed, taking its traditional non-modified 'counterpart' into consideration.

Legislation for novel foods has been adopted in the Netherlands in 1993. The

regulation endorses recommendations that foods, obtained by means of recDNA technology, should be evaluated on the basis of a case-by-case approach using a decision-tree system in which toxicity tests tend to predominate [7]. The decision trees are mainly those of the International Food Biotechnology Council (IFBC) proposal [8], with an extra one for products of animal origin. The Dutch variant stipulates an exact characterisation of the DNA insert and pays special attention to effects of novel foods on the nutritional status of the consumer. Related to this, marker genes coding for antibiotic resistance when incorporated in a stable way, are deemed admissible. However, the most important difference between the Dutch decision tree for transgenic food crops and the IFBC counterpart is that the former demands a 90 day feeding trial for each new product. The Dutch decision tree exempts only those foodstuffs that have the place of insertion characterised to such an extent that it can be established that no deleterious effects to the metabolism occur that may affect safety as food.

It is obvious that case studies are needed which results may be valuable in designing a science-based, practically and internationally harmonised strategy for the safety assessment of novel foods of vegetable origin.

### SAFETY EVALUATION OF *Bacillus thuringiensis*(Bt)-TOXIN TOMATOES

In 1991 an EU co-sponsored research project was initiated within the framework of FLAIR (Food-linked Agro-Industrial Research) entitled '*Opportunities of transgenic food crops for the consumer and the food industry in the Community*' with the object to design a test strategy for the food safety evaluation of transgenic food plants. The project concerns the molecular, biochemical and toxicological characterisation of insect-resistant tomato transformants obtained from parental tomato lines by *Agrobacterium* mediated transformation, encoding an insecticidal crystal protein CRYIA(b) from *Bacillus thuringiensis* (for the classification of the *B. thuringiensis* toxins see [9]). An easily detectable marker gene, encoding neomycin phosphotransferase (NPTII) is co-introduced into the plant genome for selection purposes.

Partners in the project are Plant Genetic Systems N.V., Ghent, Belgium (coordinator); RIKILT-DLO, Wageningen, The Netherlands in cooperation with the Agricultural University of Wageningen, and with the University La Tuscia (Viterbo, Italy); SME Ricerche, Piana di Monte Verna, Italy, and the University of Genova, DIBE, Genova, Italy.

Results of the food safety assessment of Bt-toxin tomatoes have been communicated earlier at several international meetings [10-12].

### EXPERIMENTAL STRATEGY OF FOOD SAFETY TESTING

The food safety evaluation of the transgenic Bt-toxin tomatoes was designed in such a way that answers could be given to apposite questions:

- (i) Does the CRYIA(b) protein exert a similar toxic action in mammals as observed in target insect species? The CRYIA(b) protein possesses enterotoxic effects on larvae of *Lepidoptera* insects such as *Heliothis armigera*, *Manduca sexta*, *Heliothis virescens*, and *Phthorimaea operculella*. CRYIA(b) appears to exert its toxic action in larvae, through specific interactions with the brush border membrane of midgut epithelial cells [13-15]. As binding seems essential to the onset of toxicity [16,17], the *in vivo* and *in vitro* binding of CRYIA(b) protein to gastro-intestinal (G.I.)-tract tissues

has therefore been investigated in mammals.

- (ii) Do newly introduced proteins cause systemic adverse effects in mammals, in particular putative immunotoxic (allergenic) effects, and if so, can a No Effect Level be established? Studies of the histopathology and mode of action of CRY proteins on mammals and other non-target species are very limited in number and have all used various crude spore-crystal mixtures of Bt strains [13, 18-20]. Many Bt strains produce more than one CRY protein, which complicates the elucidation of structure-toxicity relationships. On the other hand, CRYIA(b) represents a fragment of a domain of the Bt2 protoxin, that determines insect specificity and toxicity, which has not yet been subjected to a detailed safety analysis. Tests were done with single doses of CRYIA(b) protein to determine the digestability and acute toxicity, as it has been shown that Bt toxin may resist proteases [21]. Systemic effects of the protein upon passage through the gut wall, including adverse immune reactions, were tested by feeding rodents daily doses of CRYIA(b) protein for 30 days.
- (iii) Does the applied recDNA technology induce alterations in metabolic functions, which lead to significant changes in the nutritional composition of the genetically modified tomato, or in the content of naturally occurring toxicants (i.e. glycoalkaloids), which could negatively influence the safety of the product as food? Thereto, chemical analyses were made of selected macro- and micronutrients and of the most common glycoalkaloid in tomato,  $\alpha$ -tomatine. In addition, a 90 day feeding trial was conducted with rats using diets containing 10% (w/w) of lyophilised powder from whole Bt-toxin tomatoes and control fruits.

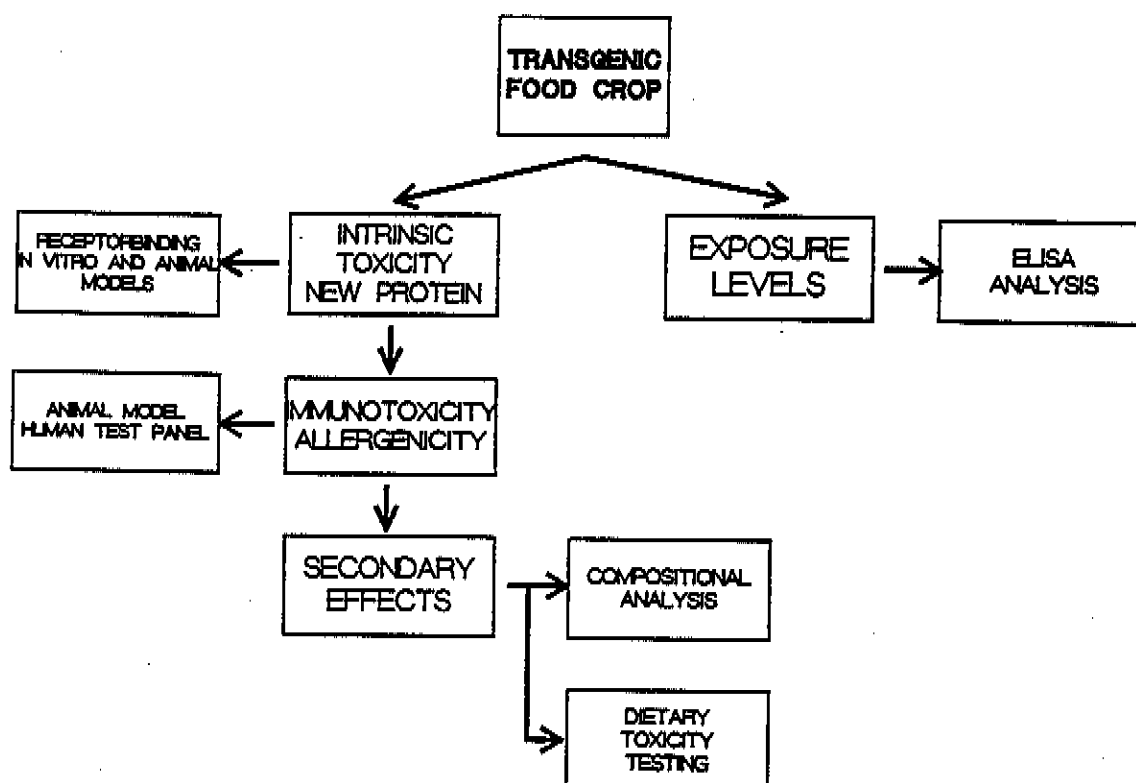
An overviewing summary of the scientific data to be generated in relation to safety evaluation of (a) CRY protein(s) in genetically modified plants is depicted in figure 1.

#### CRYIA(b) protein and Bt-toxin tomato lines

To produce milligram quantities of purified CRYIA(b) protein, the Bt2 gene was cloned from *B. thuringiensis* var. *berliner* 1715 [13,22] which encoded the 130 kDa  $\delta$ -endotoxin (Bt2-protoxin). After purification and solubilisation from the *E. coli* strain K514 (pGI502) the protoxin was digested with trypsin and chymotrypsin to yield the active toxic fragment CRYIA(b) (Mr 66-68 kDa). Subsequent purification and characterisation of the recDNA CRYIA(b) protein was carried out as described previously [13].

Transgenic tomato plants were obtained from the parental line TL001 (a round type tomato) by *Agrobacterium* mediated transformation using cotyledons as the explant. A disarmed *Agrobacterium* strain containing the vector pPSO216 was used for transformation. The vector pPSO216 comprised two chimeric genes between the T-DNA border repeats. The chimeric *neo* gene consisted of the promoter of the T-DNA TR1' gene (PTR1'), the coding region of the *neo* gene encoding neomycin phosphotransferase II (NPTII) from transposon Tn5 and the 3' untranslated end from the octopine synthase gene (3'ocs) (16). The construction of the chimeric *pnos-neo-3'ocs* gene has been described by Hain et al. [23]. The chimeric Bt gene consisted of the woundstimulated promoter of the T-DNA TR2' gene (PTR2'), the coding region of the C-terminal truncated Bt2 gene, called *IAb6* derived from the coding region bt884 [22], and the 3' untranslated end from the T-DNA gene 7 [24]. Based on entomological and agronomical criteria the transformant RLE13-0009 was chosen for further characterisation.

Figure 1. Testing Elements for Safety Assessment of Bt Food Crops



### Insecticidal activity of CRYIA(b) in mammals

Male Brown Norway rats (n=4) were fed CRYIA(b) protein in 5 g of standard feed, corresponding to a human daily consumption of approximately 2,000 kg of transgenic Bt-toxin tomatoes, assuming a level of 60 µg CRYIA(b)/kg of fresh field tested tomatoes. Seven hours after administration the animals were sacrificed for immunocytochemical analysis of the CRYIA(b) binding to G.I.-tract derived tissues [25]. For the detection of CRYIA(b) protein a polyclonal antiserum was used according to Höfte et al. [22]. No binding of CRYIA(b) protein could be detected in tissue segments taken from the oesophagus, stomach, duodenum, ileum, jejunum and colon. Moreover, no histopathological damage could be observed in G.I.-tract derived tissues from treated animals.

The *in vitro* binding of CRYIA(b) to intestinal tissue sections of mice, rats, Rhesus monkeys and humans has been studied by modifying the immunocytochemical method as described by Bravo et al. [26]. The immunocytochemical analysis of CRYIA(b) in intestinal tissue sections of rat, Rhesus monkeys and man was carried out using the corresponding monoclonal antiserum (mouse Ig). It appeared necessary to use a polyclonal antibody

against CRYIA(b) (rabbit Ig) in mice tissues, in order to avoid cross reaction of the second antibody to IgA present murine tissues of the small intestine. In epithelial cell layers of segments of esophagus, stomach, duodenum, jejunum, ileum and colon tissue of mice and rats, no specific binding of CRYIA(b) protein was found. Whereas, an uniform binding of the protein was observed over the entire length of the brush border epithelium in the midgut of larvae of *Manduca sexta*. Similar experiments were performed with intestinal tissues of Rhesus monkeys and humans, where no specific binding of CRYIA(b) protein could be detected. Except, a positive staining in caecum and colon tissue of Rhesus monkeys, which could be reduced, however, by prolonged incubation with methanol containing 0.6% (v/v)  $H_2O_2$  (i.e. inhibition of the endogenous peroxidase activity), indicating an aspecific effect.

#### **Digestability of the CRYIA(b) protein**

The degradation of CRYIA(b) protein was studied under simulating human gastrointestinal conditions [27]. Incubation of the protein under conditions of pH 2, in the presence of pepsin (1 : 100, w/w), and subsequently at pH 8, in the presence of chymotrypsin and trypsin (1 : 25, w/w), revealed upon analysis by gel permeation HPLC chromatography, an extensive fragmentation of the proteins to peptides with molecular weights below 10 kDa. However, the degradation of the CRYIA(b) protein is clearly a two-step process. After two hours at pH 2 in the presence of pepsin the protein was readily cleaved to yield a 15 kDa fragment, and successively to smaller fragments ( $< 10$  kDa) after continued treatment with chymotrypsin and trypsin at pH 8. The NPTII protein on the contrary appeared completely digested at pH 2 in the presence of pepsin.

The digestability of CRYIA(b) protein was studied in male Brown Norway rats (n=5), which were fistulated in the ileum. Animals were fed CRYIA(b) in 5 g of standard feed, corresponding to an approximate human daily consumption of 2,000 kg transgenic Bt-tomatoes. Chymus was collected after 5-7 hours, and SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis revealed no intact CRYIA(b) protein, but fragments of 20-30 kDa and smaller. In samples taken after 7 hours no large fragments of CRYIA(b) were visible, indicating a further extensive degradation of the protein during G.I.-tract passage into polypeptides smaller than  $< 9$  kDa.

#### **Short-term oral toxicity of CRYIA(b) in rodents**

CRYIA(b) protein was administered to female NMRI mice (n=10/dose group) via drinking water *ad lib.*, at two dose levels during 28 days. The highest dose level corresponded to a daily consumption of 500 kg of transgenic Bt-toxin tomatoes. Control mice were administered tap water and test solutions were refreshed every third day. There were no changes observed in body weight gain, absolute and relative organ weights, and hematological values, including white blood cell differential counts, between treated and control animals. Furthermore, histopathology of the G.I.-tract of animals treated during 28 days failed to indicate any signs of damaging effects caused by CRYIA(b) protein.

New Zealand White male rabbits (n=4/dose group) were dosed via drinking water *ad lib.* at a concentration of 0.75 mg CRYIA(b)/L during 31 days. Controls received tap water and test solutions were refreshed each day. Data of average water consumption indicated a daily intake of CRYIA(b) protein corresponding to a human daily consumption of 60 kg transgenic Bt-toxin tomatoes. There were no differences observed in food consumption and water uptake nor in body weight gain and absolute and relative liver and kidney weights between treated and non-treated animals. No changes were noted in

hematological parameters, including leucocytes differential counting. Histopathology of various segments of the G.I.-tract did not reveal any harmful effect in treated animals compared to controls. Analysis of serum, sampled at day 14 and 28 after initiation of the experiment, did not indicate that in treated rabbits antibodies were raised against CRYIA(b). With respect to immunotoxic reactions, no significant differences were found in the total immunoglobulin (IgG) content of serum of treated animals compared to that of controls.

#### **Hemolytic effects of CRYIA(b) protein**

Human red blood cells (RBC) were tested for the hemolytic potential of CRYIA(b) by incubation of the RBCs with the protein, and monitoring the osmotic fragility. No hemolysis was observed. Since it has been postulated that the site of interaction of CRY proteins may be the ATPase, which is located on the cytoplasmic side of the membrane [19], CRYIA(b) was entrapped inside the RBC by the method of hypotonic dialysis [28], and subsequently tested for its hemolytic potency. Erythrocytes containing CRYIA(b) showed negligible hemolysis comparable to that observed with RBCs swollen in the absence of CRYIA(b) protein or of RBCs entrapped with albumin.

#### **Chemical analysis of nutrients of Bt-toxin tomatoes**

Field tested mature tomatoes were harvested, lyophilised, and representative samples were analysed for compositional changes under conditions corresponding to the expected time for processing (i.e. soon after harvest). Selected macro- and micronutrients of the transgenic line RLE13-0009 were compared to the nutritional composition of its respective nontransformed parental line TL001. The compositional values of transgenic Bt-toxin tomatoes were within the typical ranges determined for nontransformed tomatoes, and found to be within published ranges [29]. It is important to note that nutritional components normally vary due to both cultivar-related and environmental-related influences. No significant differences were observed in the concentrations of  $\alpha$ -tomatine between mature tomatoes of modified plants and controls (range: 1.4 - 1.7 mg  $\alpha$ -tomatine/kg fresh weight of tomatoes). Other known solanaceous alkaloids (i.e. solanine) were not detected. However, the extraction procedures developed to isolate and quantitate the glycoalkaloids in potatoes [30] did not prove to be adaptable for use in tomatoes and had to be modified.

#### **90-Day feeding trial with Bt-toxin tomatoes**

The field tested transgenic Bt-toxin tomato line RLE13-0009 and its respective control line TL001 were selected for a 90 day feeding study in rats. The field trial manifested no significant differences in vegetative growth and harvest characteristics between transgenic tomatoes and controls. In the harvested transgenic tomato variety RLE13-0009 the CRYIA(b) protein was typically expressed in fresh tomatoes at levels of 7.5 ng/mg protein, in induced tomato fruit at levels of 25.4 ng/mg of protein and in lyophilized tomato fruit at levels of 40.6 ng/mg protein with a nominal protein content of about 0.8% of fresh weight.

Three groups of 12 male and 12 female Wistar rats (5 weeks of age) were fed during a period of 91 days, respectively, a control semi synthetic animal diet (Muracon SSP TOX), the same diet supplemented with 10% (w/w) of lyophilized tomato material of the parental line TL001, or with 10% (w/w) of lyophilized transgenic Bt-toxin tomatoes containing 40.6 ng CRYIA(b)/mg of protein. The macro- and micronutrient composition was equalised in all diets; i.e. 7.5% E(energy) fat, 20% E protein, 41.5% E carbohydrate and 10.4% fibre (w/w). The amounts of supplementary minerals and vitamins were deduced from the actual levels

found in the freeze-dried tomato fruits. The mean daily intake of tomato powder over the 91 days period corresponded to approximately 200 g of tomatoes/kg body weight of rat, which was equivalent to a daily human consumption of 13.0 kg of fresh tomatoes. There were no treatment related changes in appearance or behaviour of any study animal observed under the conditions of this investigation. There was no feed refusal, however, a statistically significant higher daily water uptake was recorded in tomatoes fed animals. No significant differences in serum chemistry, hematology, and urinalysis values were noticed between the different diet groups. All animals survived the full course of treatment and all necropsies were performed according to the protocol schedule. Gross examination of all animals failed to uncover any significant macroscopic abnormality. The weights of for instance liver, kidneys, spleen, and thymus, expressed as percent of body weight, did not show differences in transgenic Bt-toxin tomato fed animals compared to controls. Microscopic examination included in this study did not reveal signs of pathologic effects related to transgenic Bt-toxin tomato feeding. The microscopic lesions found in the Bt-toxin tomato fed animals were either found to a similar degree in control animals or are commonly encountered spontaneous lesions enzootic among Wistar rats. The results of the 91 days feeding trial in rats obtained up till now are reassuring with respect to the food safety of the insect resistant transformant RLE13-0009.

### TOXICOLOGIC CONSIDERATIONS

In case of insect-resistant Bt-toxin tomatoes the encoded product is a novel (i.e. foreign) food protein which necessitated a toxicologic evaluation. In establishing the safety of the CRYIA(b) protein an approach was followed which was in keeping with guidelines for additives (e.g. JECFA). The given example of insect-resistant Bt-toxin tomatoes showed the necessity of such an approach. Extensive testing of the CRYIA(b) protein revealed that its specific insecticidal action does not occur in mammals. *In vivo* and *in vitro* binding experiments in intestinal tissues from rodents, Rhesus monkeys and humans indicated the absence of specific binding sites for the protein. Even at exaggerated dose levels, corresponding to 2,000 kg of fresh transgenic Bt-toxin tomatoes, no adverse effects could be observed in rodents, and no evidence was found for immunotoxicity of the CRYIA(b) protein. Beside knowledge of toxicity and exposure levels of the encoded proteins, the evaluation should also focus on the potency of secondary effects on plant metabolism, which may be detrimental to food safety.

Related to this latter aspect, a 90 day feeding trial with complex novel foods as a substantial part of the animal diet may pose, however, problems with regard to the conventional testing procedures for e.g. additives, because:

- apparent toxic effects may result from nutritional imbalances caused by large quantities of test material to the basal rodent diet, rather than from the inherent toxicity of the transgenic food crop. For example in the present study, the microscopic examination revealed a calcareous debris situated at the corticomedullary junction of female kidneys. Although, a slightly higher incidence of focal intratubular lithiasis was found in the transgenic Bt-toxin tomato fed females, these findings are believed to be of toxicologic insignificance. Because mineral deposits are often found within the renal tubules of the laboratory rat, and are also relatively common in the urinary bladder and renal pelvis. It is seen in young rats, predominantly in females, and their presence correlates with the calcium/phosphorus ratio in the diet, but carbonic anhydrase inhibitors may also precipitate the deposition

- of calcium salts in the urinary tract under certain dietary conditions [31],
- the safety testing of complex food products in rodents may offer cumulative toxicity as a means of detection, and
- in rodents the administration of multiples of expected exposure levels may be virtually impossible.

In order to avoid some of above mentioned disadvantages the application of a semi-synthetic rodent diet with interchangeable nutrients, adapted for the nutritional requirements of rats and deduced from the actual levels in transgenic Bt-toxin tomatoes, was studied. Although the given example demonstrated the feasibility of this approach, in view of the diversity of varieties and the risks dealt with, the choice of an analytical approach as customarily performed to screen for possible secondary effects in transgenic plants appears to be more justified. An analytical approach based on single component analysis has its limitations, however.

With respect to safety evaluation of Bt-toxin tomatoes other aspects have still to be investigated, such as:

- (i) The posttranslational modification of CRYIA(b) protein obtained from fermentations with recombinant bacterial strains may be different from the one expressed in transgenic food plants. Differences, which possibly results in a modified toxic profile and immunogenicity. On the other hand, the lack of posttranslational modification could also induce toxicity.
- (ii) The long term use of Bt spore preparations in the field as insecticides has not uncovered evidence for allergic reactions in workers. Despite this, studies on the allergenic potency of CRYIA(b) should be foreseen, in particular because of the potential exposure of the general population to Bt-toxins via transgenic plants. However, testing systems to investigate the allergenic potency of foreign food proteins are not yet available. A major problem in the development of useful and validated test systems is the considerable variation in sensitivity to sensibilisation and subsequent allergic reaction in individuals.

## REGULATORY CONSIDERATIONS

As generally recognised the novel food should be no less safe nor should it be expected to be safer. As yet, considering regulatory aspects of foodstuffs derived from genetically modified plants a case-by-case approach seems to be the most efficacious strategy. Complex foodstuffs require an apprehensive problem-solving approach rather than rigid testing according to protocols. A case-by-case approach allows to include the latest scientific findings in the evaluation, thereby guaranteeing maximum safety of the novel food. However, it may require more efforts to safeguard uniformity of evaluation. In any case, the criteria for the evaluation of newly expressed proteins in transgenic plants are largely comparable to those used for food additives. In case of transgenic plants the identity of the non-transgenic control plant must be known, and the host plant should have a history of safe use. The DNA insert must be fully characterised and located, and devoided of any toxic element harmful to humans. The wholesomeness of transgenic food crops must be based on comparison with its parental line(s) as well as to varieties in the wider sense.

It is most likely that methods to analyse nutrients, naturally occurring toxicants as

well as antinutritional factors will be used in order to identify possible secondary metabolic changes in plants as a result of foreign gene insertion, rather than animal feeding trials. Information on the composition of most food crops and derived products is fairly limited. Also knowledge on the variation of components in different varieties and in different environments or stages of development is still in its infancy. New ways of chemical analysis should therefore be explored, focusing at the characterisation of plant extracts with respect to 'metabolic fingerprints' rather than isolation and structural characterisation of single compounds. This analytical approach offers possibilities to refrain from animal feeding trials with whole foods. Metabolic fingerprinting, determining compositional patterns of the whole food product using techniques such as NMR, LC(-GC)/MS(-MS) and CZE(-MS), may be a good alternative to traditional chemical analysis. Moreover, databases should be set up to supplement existing data in relation to food components with the purpose to gain more insight into both the composition of plant varieties and the variation in composition depending on variety, stage of cultivation or climate conditions. Next to the method of metabolic profiling, a differential analysis at the DNA and/or mRNA level would be, at least in theory, of great value. However, fundamental knowledge on the functioning mechanisms at DNA level is largely lacking. In case these new analytical approaches can not generate sufficient data to establish safety or if they indicate significant compositional differences compared with traditional 'counterpart' crops, additional animal feeding trials can still be considered to assess safety of the transgenic food plant.

## CONCLUSIONS

The safety assessment of transgenic insect resistant Bt-toxin tomatoes up till now indicate that:

- No specific receptors for CRYIA(b) protein are present along the G.I.-tract of mammals including man. Furthermore, no histopathological effects of the protein have been observed in the digestive mucosa cells lining the mammalian G.I.-tract.
- CRYIA(b) and NPTII degrade rapidly under simulating G.I.-tract conditions to smaller fragments with molecular weights below 10 kDa, and CRYIA(b) is upon high dosage oral feeding to rats digested extensively to smaller peptides.
- CRYIA(b) administered orally to mice and rabbits, does not exert signs of systemic toxicity. No indications were found for immunotoxic effects as judged from the histological examination of spleen, lymph nodes and Peyer's patches of treated animals. In serum of treated rabbits no antibodies raised against CRYIA(b) were detected, nor was the total IgG concentration elevated with respect to controls.
- In vitro experiments to test for hemolytic potency of the CRYIA(b) protein yielded negative results.
- No changes occurred in the chemical composition of transgenic tomatoes as a result of the insertion of foreign genes. It should be noted that nutrients of plants normally vary due to both cultivar-related and environmental influences. Moreover, levels of the naturally occurring toxicant  $\alpha$ -tomatine were similar in modified and control tomatoes.

- Transgenic Bt-toxin tomatoes fed to rats during 91 days as lyophilized powder mixed through the diet, did not exert any signs of adverse effects. The estimated mean intake of transgenic tomatoes during the test period corresponded to a daily human consumption of 13 kg of fresh weight tomatoes. Food intake, body and organ weights, and clinical parameters were normal, and gross and histopathological examination of organs and tissues failed to uncover toxic effects.

To summarise, in assessing the safety of transgenic plants as food following elements must be issued: (i) the specific genetic modifications involved; (ii) the characteristics of DNA inserts and expected gene products; (iii) the role of expression products in physiological processes; (iv) possible secondary metabolic changes occurring as a result of gene modification, and (v) levels of exposure to be expected in various processed foods.

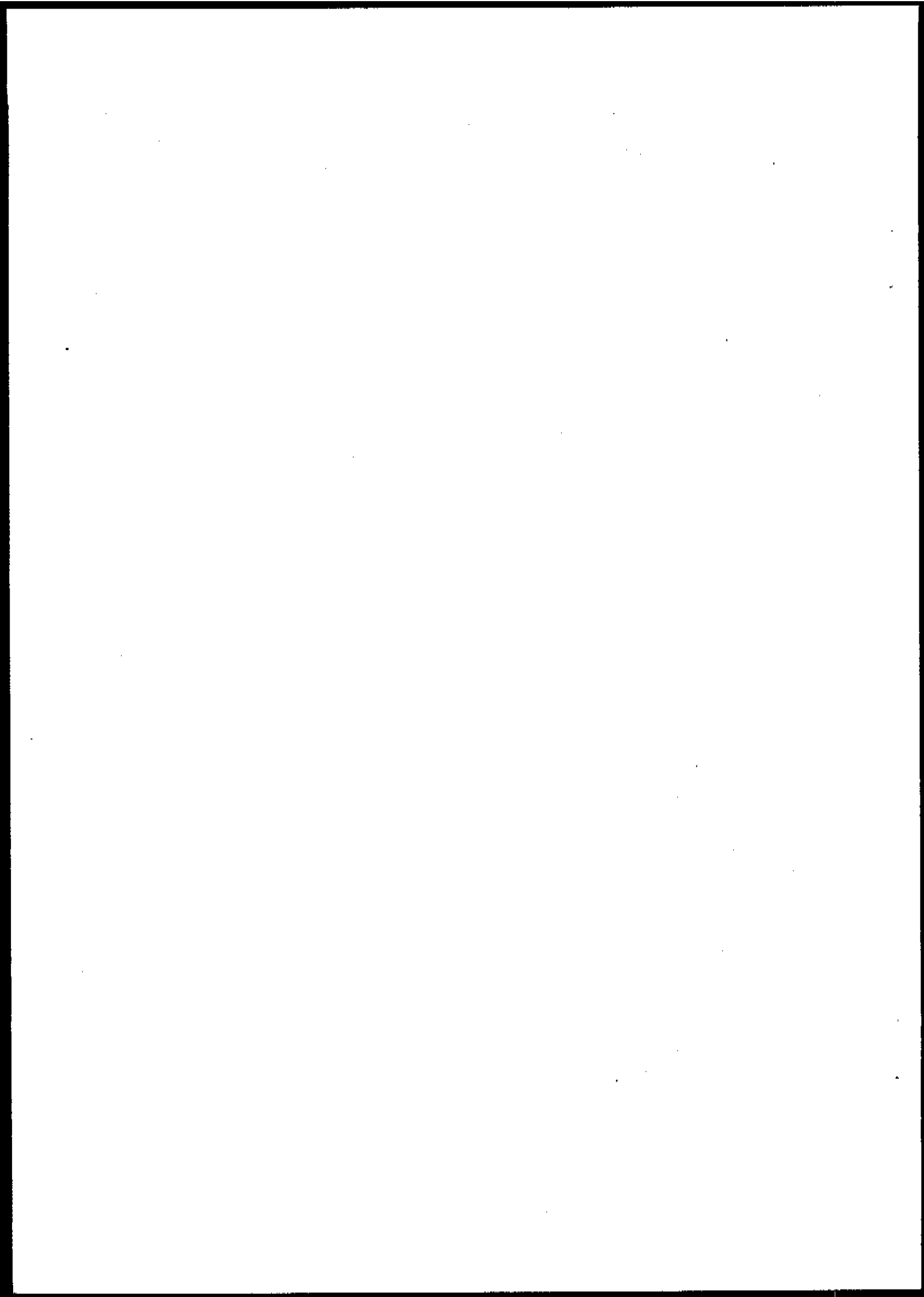
It is emphasised that the risk assessment of novel foods should take place within a general and flexible framework with case-by-case variations. The challenge for modern toxicology is to design such flexible and tailor-made evaluation strategies for transgenic food plants, taking both the mode of action of introduced proteins and the often long history of safe use of the traditional 'counterpart' into account. New analytical approaches combined with *in vitro* toxicologic test systems have great opportunity to achieve these objectives.

### Acknowledgments

We thank Dr A. Reynaerts and Dr B. Verachtert of Plant Genetic Systems for excellent scientific collaboration and for provision and biochemical characterisation of test materials and methods; M. Pensa of SME Ricerche, for providing the transgenic tomatoes and controls, and data on the nutritional composition; Prof. L. Zolla, University La Tuscia, for carrying out the hemolysis studies; M. Peters and G. van Tintelen, Centre for Small Laboratory Animals, and Dr G.M. Alink and J.H.J. van den Berg, Agricultural University, for their assistance with the animal experiments; M.E. Bienenmann-Ploum, J.F. Labrijn, G.J.M. Loeffen, G.D. van Bruchem, H.J. van Egmond, A. de Koning, A.R.M. Hamers, M.B.M. Huveneers-Oorsprong and H.M. van de Putte, RIKILT-DLO, for toxicological, histological, clinical and chemical analysis; Dr M.J. Groot and J.S. Ossekoppele, RIKILT-DLO for the gross examination and histopathology. This work was carried out in the framework of the EU Food-Linked Agro-Industrial Research (FLAIR) program (contract no. AGRF-CT90-0039).

## References

1. Fraley, R. *Bio/Technology* 1992, 10, 40-43.
2. Gadani, F. et al. *Arch. Virol.* 1990, 115, 1-21.
3. Jones, J.L. *Trends in Food Science & Technology* 1992, 3, 54-59.
4. Lindsey, K. *Agro-food-Industry, Hi-tech* 1991, 4, 8-16.
5. Oxtoby, E. *Tibtech* 1990, 8, 61-65.
6. Perlak, F.J. et al. *Proc. Natl. Acad. Sci. USA* 1991, 88, 3324-3328.
7. Food and Nutrition Council: Committee on Biotechnology of the Food and Nutrition Council (1993) *Advisory Report on Biotechnology (summary)*. Dutch Food and Nutrition Council Publications. The Hague, The Netherlands.
8. International Food Biotechnology Council (1990) *Biotechnologies and food: Assuring the safety of foods produced by genetic modification. Regul Toxicol Pharmacol* 12:S1-196.
9. Höfte, H.; Whitely, H.R. *Microbiol. Rev.* 1989, 53, 242-255.
10. Noteborn, H.P.J.M. et al. *Med. Fac. Landbouww. Univ. Ghent, MFLRA3* 1994, 59(4a), 1765-1774.
11. Noteborn, H.P.J.M. et al. In: *Proceedings 6th European Congress on Biotechnology, 1994*, (Alberghina, L., Frontali, L., Sensi, P. eds.), Elsevier science, 1045-1048.
12. Noteborn, H.P.J.M. et al. *Toxicology Letters*, 1994, 74(Suppl. 1), 58.
13. Hoffmann, C. et al. *Proc. Natl. Acad. Sci. USA* 1988, 85, 7844-7848.
14. Van Rie, J. et al. *Science* 1990, 247, 72-74.
15. Van Rie, J. et al. *Appl. Environ. Microbiol.* 1990, 56, 1378-1385.
16. Wolfersberger, M.G. *Experientia* 1990 46, 475-477.
17. Ferré, J. et al. *Proc. Natl. Acad. Sci. USA* 1991, 88(12), 5119-5123.
18. Knowles, B.H.; Ellar, D.J. *Biochim. Biophys. Acta* 1987, 924, 509-518.
19. English, L.H.; Cantley, L.C. *J. Biol. Chem.* 1986, 261, 1170-1173.
20. Nishiitsutsuji-Uwo, J.; Endo, Y.; Himeno, M. *Appl. Ent. Zool.* 1980, 15, 133-139.
21. Bietlot, H. et al. *Biochem. J.* 1989, 260, 87-91.
22. Höfte, H. et al. *Eur. J. Biochem.* 1986, 161, 273-280.
23. Hain, L. et al. *Mol. Gen. Genet.* 1985, 199, 161-168.
24. Velten, R.E.; Schell, J. *Nucleic Acid Res.* 1985, 13, 6981-6998.
25. Bravo, A. et al. *J. Invertebr. Pathol.* 1992, 60(3), 247-253.
26. Bravo, A.; Jansens, S.; Peferoen, M. *J. Invertebr. Pathol.* 1991, 60(3), 237-246.
27. Gauthier, S.F.; Vachon, C.; Savoie, L. *J. Food Sci.* 1986, 51, 960-964.
28. Zolla, L. et al. *Biochim. Biophys. Acta* 1990, 1024, 1-9.
29. *Food Composition and Nutrition Tables 1986/87*; Souci, S.W.; Fachmann, W.; Kraut, H., Eds.; Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1986, pp 694-695.
30. Van Gelder, W.M.J.; Vinke, H.; Scheffer, J.J.C. *Euphytica* 1988, 39, 147-158.
31. *Rat Histopathology* (Greaves, P.; Faccini, J.M. eds.) 1992, Elsevier, 171-172.



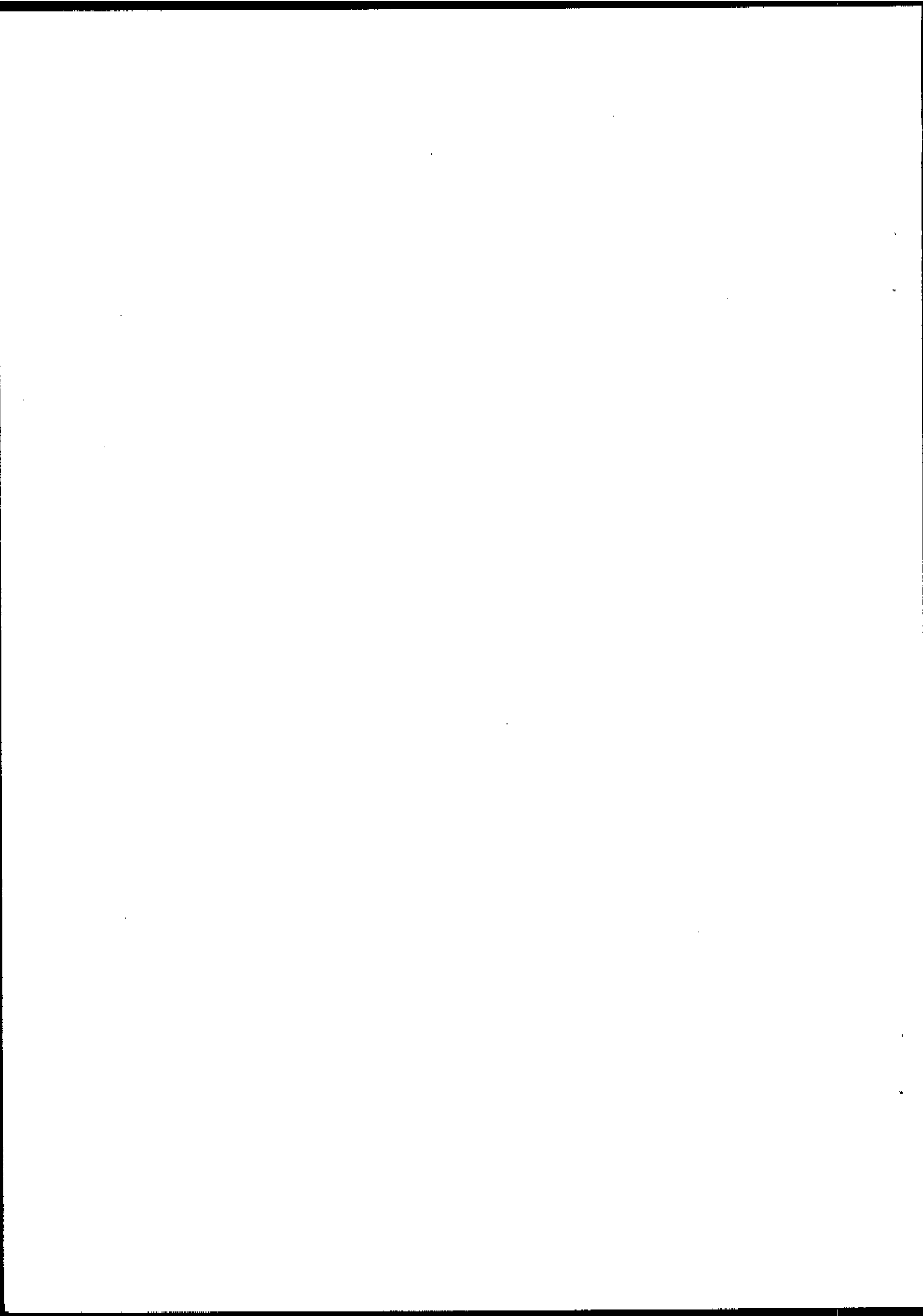
CASE STUDY 4

**Safety Evaluation of Colorado Potato Beetle-Protected Potatoes**

Roy L. Fuchs<sup>1</sup>, Glennon J. Rogan<sup>1</sup>, Pamela J. Keck<sup>1</sup>,  
Steve L. Love<sup>2</sup> and Paul B. Lavrik<sup>1</sup>

<sup>1</sup>Monsanto Company, Chesterfield Parkway North  
St. Louis, MO, USA

<sup>2</sup>University of Idaho, College of Agriculture  
Aberdeen, ID, USA



## Safety Evaluation of Colorado Potato Beetle-Protected Potatoes

Roy L. Fuchs<sup>1</sup>, Glennon J. Rogan<sup>1</sup>, Pamela J. Keck<sup>1</sup>,  
Steve L. Love<sup>2</sup> and Paul B. Lavrik<sup>1</sup>

<sup>1</sup>Monsanto Company, Chesterfield Parkway North  
St. Louis, MO, USA

<sup>2</sup>University of Idaho, College of Agriculture  
Aberdeen, ID, USA

### Abstract

Russet Burbank potato varieties have been developed that are protected season long against damage by the Colorado Potato Beetle (CPB). These plants express low levels of two new proteins, the protein from *Bacillus thuringiensis* subsp. *tenebrionis* which confers protection against CPB and the neomycin phosphotransferase protein that served as the selectable marker in the plant transformation process. Both proteins were shown to be safe for human consumption. Extensive analytical information on tuber composition established that the potato tubers produced from the CPB-protected potatoes were equivalent in composition and safety to the Russet Burbank potatoes currently in commerce. This safety evaluation verified the value of the substantial equivalence approach in assessing the safety of foods derived from genetically-modified plants. Evaluation of the foods derived from five additional genetically-modified plant products showed these products are also substantially equivalent in composition and safety to their traditionally-bred counterparts. The extensive data presented for these six products established the broad applicability of the principles of substantial equivalence in evaluating the safety of these foods.

### Introduction

Numerous genetically engineered plant products have been extensively field tested and are nearing market introduction. The first of these products, the Flavr-Savr<sup>TM</sup> tomato, was recently introduced in the United States after completing review with the appropriate regulatory agencies. Confirming the safety of the genetically engineered products and satisfying the appropriate regulatory authorities is an

important and often time-limiting step in the commercialization of these products. A case study using the safety evaluation of Colorado potato beetle-protected potatoes will be used to illustrate the detailed technical assessment employed to establish that the potatoes produced by this new variety are substantially equivalent to current Russet Burbank potato varieties.

We have carefully followed the guidance provided by the Food and Drug Administration (US FDA, 1992), the Organization for Economic Cooperation and Development (OECD, 1992) and other organizations in assessing the food safety of Colorado potato beetle-protected potatoes. This assessment focused on: (1) the introduced protein that confers insect protection and the marker gene protein used in the introduction of the gene for insect protection; and (2) the composition of important nutrients and anti-nutrients compared to that of traditional Russet Burbank potatoes. The results led to the conclusion that the potatoes produced by the Colorado potato beetle-protected variety are substantially equivalent in composition and safety to the potatoes currently in commerce. Detailed protein safety and compositional analysis of five other genetically engineered crops that are near commercialization has verified that the products from these genetically engineered crops are substantially equivalent to the corresponding products developed by traditional plant breeding.

### Background

Colorado potato beetle (CPB) is the most damaging pest of the \$2.3 billion U.S. potato crop (Casagrande, 1987; National Potato Council, 1992). CPB causes significant damage in the majority of the North American potato production regions. If untreated or poorly managed, CPB can reduce yields by up to 85% and devastate potato production (Hare, 1980; Ferro *et al.*, 1983; Shields and Wyman, 1984). Current control of CPB primarily involves the use of insecticides that are variably effective due to environmental factors and insect susceptibility. These products also significantly reduce field populations of beneficial insects which help to control other potato pests. These pesticides are expensive, with costs that can exceed \$200 per acre per season (Ferro and Boiteau, 1992).

### Development of Colorado Potato Beetle-Protected Potatoes

Potato varieties that effectively control the CPB without the use of chemical insecticides have been developed through the production of potato plants that produce an insect control protein which selectively controls this beetle without affecting non-target insects, humans or animals. These varieties were produced by inserting and expressing a gene from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) in the potato plant (Perlak *et al.*, 1993) using *Agrobacterium*-mediated transformation (Newell *et al.*, 1991). The CPB-protected plants produce low levels of two new proteins, the *B.t.t.* protein for protection against CPB and the neomycin phosphotransferase II (NPTII) protein produced to enable the selection of cells expressing the *B.t.t.* protein in tissue culture. The *B.t.t.* protein produced is identical to one of the insecticidal proteins contained in several commercial microbial formulations that have been used safely since 1988 (EPA, 1988; EPA, 1991). The *B.t.t.* protein is selectively active against a narrow spectrum of coleopteran insects

(MacIntosh *et al.*, 1990). Upon ingestion by susceptible insects, feeding is inhibited with disruption of the gut epithelium, which results in the eventual death of the insect pest (Slaney *et al.*, 1992).

Over 80 field trials conducted over the past four years with CPB-protected potatoes have demonstrated effective control of all stages of the CPB pest. Control, without insecticide application, was comparable or superior to that observed for control plants treated with insecticides on a regular schedule (Perlak *et al.*, 1993).

These potatoes offer advantages to the grower, the consumer and the environment for controlling this devastating insect pest. The superior control demonstrated for both the larval and adult will enable growers to significantly reduce the amount of chemical insecticide now applied to their crop while maintaining comparable yields. Elimination of the need for chemical insecticides to control CPB also will reduce the fuel, time and costs required by the grower to apply these insecticides. Reducing the amount of insecticide applied will aid in the implementation of Integrated Pest Management (IPM) practices as beneficial insect populations are maintained and allowed to increase, which significantly contributes to the reduction of other potato pests not directly controlled by the CPB-control protein. The *B.t.t.* protein has been shown to be safe to nontarget species, including humans (EPA, 1988, 1991) and thus provides an environmentally safe and compatible means to control CPB. In addition, CPB-protected potatoes will benefit both large and small growers since no additional labor, planning or machinery is required for adoption.

### **Food Safety Evaluation**

As these CPB-protected varieties have been developed, extensive safety evaluation has been performed which has encompassed both food and environmental safety. However, this case study will focus on the food safety assessment, since the scope of this workshop is assessing the substantial equivalence of the foods derived from genetically-modified plants and their traditional counterparts. The agronomic and morphological characteristics of the genetically-modified plant have confirmed the efficacy and stability of the introduced trait and the lack of significant unintended effects that may be attributable to the transformation process.

The safety evaluation of CPB-protected potatoes will be described in four components. The first component was to evaluate the morphological and agronomic characteristics of the CPB-protected lines, selecting those lines that met specific performance standards for commercial potato varieties. The second component consisted of molecular characterization of the inserted genes and gene products. Thirdly, the safety of the newly expressed proteins was assessed. And finally, the composition of important nutrients and anti-nutrients of potatoes produced in the tubers of both the CPB-protected potato and the Russet Burbank potatoes currently in commerce was evaluated to determine if the potatoes from the new lines were substantially equivalent to potatoes in commerce. Substantial equivalence is the term described by the Organization for Economic Cooperation and Development (OECD, 1992) and embodies the concept that if the food from two varieties are

substantially equivalent, then the food for the new variety can be treated in the same manner as the food from the parental variety with respect to safety.

#### *Morphological and agronomic evaluation*

Hundreds of genetically engineered potato lines of CPB-protected potatoes have been produced and evaluated in the laboratory, the greenhouse and the field. After several years of detailed morphological and agronomic evaluation under field conditions, seven independently transformed CPB-protected lines were selected for commercial development. All seven lines met the stringent product performance standards established for new potato varieties. Evaluations consisting of plant vigor, growth habit characteristics, yield, tuber quality and general insect and disease susceptibility have shown the seven CPB-protected potatoes to be morphologically and agronomically equivalent to the parental Russet Burbank potatoes (Perlak, *et al.*, 1993).

#### *Molecular characterization*

Detailed molecular analysis was performed on each of the seven CPB-protected lines. Binary, double border vectors were used in the plant transformation process (Perlak, *et al.*, 1993). No DNA outside the right and left borders were detected in any of these seven lines. Five lines were shown to contain a single copy of the DNA contained within the borders that typically delimit the extent of DNA transferred from the plasmid vector to the plant. One line contained two copies of DNA inserted in a head-to-tail fashion at a single site. The other line contained DNA inserted at two independent sites, with one copy of DNA, delimited by the borders, at each site.

All seven lines produced both the *B.t.t.* and NPTII proteins, as predicted from the molecular characterization. The levels of these proteins were estimated for all seven lines from samples collected from seven different field tests conducted in 1992 under standard agronomic field conditions that were representative of the environments used for commercial potato production. Protein levels were assessed in the leaf and tuber, as well as other tissues that are not of importance from a food safety perspective. Levels for the *B.t.t.* protein ranged from 5.4 to 28.3 micrograms per gram of fresh weight in the leaf tissue and 0.4 to 2.0 micrograms per gram of fresh weight in the tuber. The NPTII protein was expressed at 0.9 to 5.2 micrograms per gram of fresh weight in the leaf tissue and 0.2 to 1.0 micrograms per gram of fresh weight in the tuber. These levels of expression result in the *B.t.t.* and NPTII proteins comprising less than 0.01 and 0.005% of the protein in the potato tuber, respectively.

#### *Safety assessment of the expressed proteins*

Both the *B.t.t.* and NPTII proteins have a history of safe use. The *B.t.t.* protein expressed in these potato tubers is comparable to one of the proteins contained in the commercial microbial formulations that have been used commercially since 1988. These proteins have comparable insecticidal activities and insect specificities, both showing stringent selectivity for CPB. Both proteins share similar molecular weights and show similar immuno-reactivities. These data establish the substantial equivalence for the *B.t.t.* protein produced in potato tubers of the CPB-protected potatoes to the *B.t.t.* protein in commercial microbial products. Therefore, the

extensive safety data base developed for the commercial microbial products confirms the safety of this protein, as expressed in potato tubers.

Because the CPB-protected potato will be one of the first genetically engineered products introduced into commerce, we also repeated many of the studies performed with the microbial products to provide an additional assurance of safety. For this safety assessment, we obtained gram quantities of purified *B.t.t.* protein by expressing this protein in *Escherichia coli*. Limited expression prohibited the isolation of large quantities of this protein from the potato tubers directly. However, minor (microgram) amounts of this protein were purified from the potato tuber. The *B.t.t.* protein isolated from both the potato tuber and from *E. coli* were shown to be chemically and functionally equivalent. A series of commonly used analytical assays were used for this equivalence assessment, including establishing comparable insecticidal activities, molecular weights, immuno-reactivities, N-terminal amino acid sequences and lack of post-translation modification.

An acute gavage study was conducted in mice with the *E. coli*-derived *B.t.t.* protein that confirmed its safety. Acute gavage studies were considered most appropriate since proteins that are known to be toxic act via acute mechanisms (Pariza and Foster, 1983; Jones and Maryanski, 1991; Sjoblad *et al.*, 1992). Following EPA guidelines, a dose was used that was equivalent to over a 2.5 million-fold safety factor, based on the average consumption of potato and the level of the *B.t.t.* protein present in the tuber. No adverse effects were observed in terms of food consumption, weight gain, mortality or gross necropsy. Purified protein was also used in an *in vitro* digestion experiment which demonstrated that the *B.t.t.* protein has an extremely short half-life (less than 30 seconds) under simulated gastric conditions (The United States Pharmacopeia, 1990). These studies confirm the safety of the *B.t.t.* protein for human consumption.

Likewise, the NPTII protein has been shown to present no food safety concerns. The description and safety assessment of the NPTII protein has been discussed in detail in the recent FDA processing aid food additive approval of this protein (FDA, 1994) for use in tomato, cotton and canola. The Environmental Protection Agency (EPA) also recently approved the NPTII for use as a selectable marker, when accompanying a pesticidal trait (such as the *B.t.t.* protein) (EPA, 1994). Published articles by Flavell *et al.* (1992), Nap *et al.* (1992) and Fuchs *et al.* (1993a, 1993b) also confirmed the safety of the NPTII protein. The latter two references describes acute gavage and digestive fate studies for NPTII that were comparable to those described above for the *B.t.t.* protein. No adverse affects were observed in the acute gavage study with greater than a 5 million-fold safety factor compared to projected consumption. The half-life of the NPTII protein in the simulated digestive fate study was also less than 20 seconds. Both studies confirmed the mammalian safety of this protein. A recent report from a workshop sponsored by the World Health Organization (WHO) also supported the safety of the NPTII protein (WHO, 1993).

Both proteins were shown to pose no significant allergenic concerns using the approach described by Fuchs *et al.* (1994) and Padgett *et al.* (1995). This approach

relies on the comparison of the physio-chemical properties of the *B.t.t.* and NPTII proteins to the properties of commonly allergenic proteins. The properties of commonly allergenic proteins have been described in detail by Taylor (1992) and Taylor, *et al.* (1987, 1992).

Whereas commonly allergenic proteins are typically prevalent in food, stable to the acidic and proteolytic conditions of the digestive system, stable to food processing and are glycosylated (Table 1), both the *B.t.t.* and NPTII protein share none of these characteristics. Furthermore, neither protein shares any significant amino acid sequence homology to known allergens. Although none of these biochemical criteria alone enable prediction of the allergenic potential of proteins, the combination of these characteristics provide a strong bases to conclude that neither protein poses a significant allergenic concern. The lack of any reports of sensitization to the commercial microbial formulations also supports the lack of allergenic concerns with the *B.t.t.* protein.

These studies on the *B.t.t.* and NPTII proteins clearly establish that these two proteins pose no significant safety concerns for human consumption.

#### *Composition of important nutrients and anti-nutrients*

The important nutrients and natural anti-nutrients were assessed for both the CPB-protected potato and the parental Russet Burbank potatoes to determine if both were substantially equivalent in composition. These analyses were conducted on potato tubers from both varieties which were grown under standard agronomic conditions in several geographical locations.

Levels of protein, fat, carbohydrate, dietary fiber and ash are equivalent. The levels of the important vitamins - vitamin C, vitamin B6, thiamine, niacin, folic acid and riboflavin are also equivalent. Levels of important minerals - calcium, copper, iron, iodine, magnesium, phosphorus, potassium, sodium and zinc are comparable. The only class of important potato natural anti-nutrients, the glycoalkaloids (solanine and chaconine), are also comparable in both the CPB-protected potatoes and Russet Burbank potatoes. The compilation of data established that tubers from the CPB-protected potatoes are substantially equivalent in composition to the Russet Burbank control potato (Table 2).

The analyses that were performed on the CPB-protected potatoes were considerably more extensive than the analyses which have been performed for new potato varieties produced through traditional breeding. This was considered appropriate for one of the first products generated with this technology. In the future, these analyses should focus on the key nutrients and anti-nutrients that are routinely assessed by plant breeders for new potato varieties and not include the extensive list performed in this study.

As a final confirmation of the equivalent wholesomeness of the CPB-protected potatoes, raw potato tubers from both CPB-protected and the Russet Burbank control were fed, along with the regular rat diet, to rodents in a 28-day study. No

differences in consumption, growth rates or gross necropsy were observed during these studies. These data confirmed that the CPB-resistant potatoes are equivalent in wholesomeness to the Russet Burbank potatoes.

### **Colorado Potato Beetle-Protected Potatoes are Substantially Equivalent in Composition and Safety to Russet Burbank Potato Varieties**

All the data generated in the morphological and agronomic assessment, the molecular analyses, the safety evaluation of both proteins and the composition of the CPB-protected potato confirm that the CPB-protected potatoes are substantially equivalent to the Russet Burbank control. The only difference is that this new potato variety is protected, season-long against damage by the Colorado potato beetle by virtue of the expression of the *B.t.t.* protein, which, along with the NPTII protein, was shown to be safe for human consumption.

### **Application of the principles of substantial equivalence to other genetically-engineered plant products**

The detailed approaches used to establish that the CPB-protected potatoes are substantially equivalent to current varieties of potato have also been applied to several other genetically-engineered products that are being planned for commercialization. These examples confirm the value and applicability of the substantial equivalence approach to assess the safety of genetically-engineered plant products and confirms that these products are substantially equivalent to current commercial products that have a history of safe use. Assessments similar to those described for CPB-protected potatoes have been completed for the following products: cotton protected against lepidopteran insect pests (Bt cotton), soybeans containing the Roundup Ready™ gene (RR soybean), canola with the Roundup Ready™ gene (RR canola), tomato with delayed ripening (Del Rip tomato) and cotton with the Roundup Ready™ gene (RR cotton). Extremely low levels of the proteins encoded by the introduced genes are expressed in these products. These proteins were all rapidly degraded in simulated digestive fluid studies (Table 3) and have been shown to cause no adverse effects in acute mouse gavage studies at levels exceeding one-thousand fold higher than the maximal anticipated consumption levels (Table 4).

Over 11,000 assays for approximately 450 different nutritional or anti-nutritional components for multiple lines of these six different crops (including CPB-protected potatoes) have confirmed that each of these genetically-engineered products are substantially equivalent to the non-engineered varieties used today (Table 5). Furthermore, appropriate animal wholesomeness studies with each of these products have also confirmed that there are no meaningful differences between these products and the products currently in the market.

### **Conclusions**

The data generated for the safety of both of the newly expressed proteins and the extensive compositional analyses confirmed that the tubers derived from the CPB-protected potato varieties are substantially equivalent in composition and safety to the Russet Burbank potatoes in commerce today. This safety evaluation verified the value of the substantial equivalence approach to assess the safety of these new

potato varieties. Furthermore, the broad applicability of the principles of substantial equivalence was reinforced as these principles were applied to establish the food safety of the food products derived from five other genetically engineered plant products. The extensive data presented from these six products strongly support the concepts and principles of substantial equivalence in assessing the food safety of genetically-engineered plant products.

### **Acknowledgments**

We thank the many members of the Regulatory Science and Regulatory Affairs programs that were responsible for helping to develop and implement the concept of substantial equivalence for the safety assessment of genetically engineered plant products. We also especially thank all those who helped to develop, analyze and assure the quality of the data presented in this manuscript.

## References

- Casagrande, R.A. 1987. The Colorado Potato Beetle: 125 Years of Management. *Bull. Entomol. Soc.* 33:142-150.
- Environmental Protection Agency (EPA). 1988. Guidance for the Re-registration of Pesticide Products Containing *Bacillus thuringiensis* as the Active Ingredient. U.S. Government Printing Office. NTIS PB 89-164198.
- Environmental Protection Agency (EPA). 1991. Delta Endotoxin of *Bacillus thuringiensis* variety *san diego* Encapsulated in Killed *Ps fluorescens*. *EPA Pesticide Fact Sheet*, EPA/OPP Chemical Code Number 128946-1.
- Environmental Protection Agency (EPA). 1994. Neomycin Phosphotransferase II; Tolerance Exemption. *Federal Register* 59:49351-49353
- Ferro, D.N. and G. Boiteau. 1992. Management of Major Insect Pests of Potato. In *Plant Health Management in Potato Production*. R.C. Rowe, ed. American Phytopath. Soc. Press, St. Paul, MN. p. 209-234.
- Ferro, D.N., B.J. Morzuch and D. Margolies. 1983. Crop Loss Assessment of the Colorado Potato Beetle (*Coleoptera: Chrysomelidae*) on Potatoes in Western Massachusetts. *J. Econ. Entomol.* 76:349-356.
- Flavell, R.B., E. Dart, R.L. Fuchs and R.T. Fraley. 1992. Selectable Marker Genes: Safe for Plants? *Bio/Technology* 10:141-144.
- Food and Drug Administration (FDA), Department of Health and Human Services. 1992. Statement of Policy: Foods Derived from New Plant Varieties. *Federal Register*. 57:22984-23005.
- Food and Drug Administration (FDA). 1994. Secondary Direct Food Additives Permitted in Food for Human Consumption; Food Additives Permitted in Feed and Drinking Water of Animals; Aminoglycoside 3'-phosphotransferase II. *Federal Register* 59:26700-26711.
- Fuchs, R.L., R.A. Heeren, M.E. Gustafson, G.J. Rogan, D.E. Bartnicki, R.M. Leimgruber, R.F. Finn, A. Hershman and S.A. Berberich. 1993a. Purification and Characterization of Microbially Expressed Neomycin Phosphotransferase II (NPTII) Protein and Its Equivalence to the Plant Expressed Protein. *Bio/Technology* 11:1537-1542.
- Fuchs, R.L., J.E. Ream, B.G. Hammond, M.W. Naylor, R.M. Leimgruber and S.A. Berberich. 1993b. Safety Assessment of the Neomycin Phosphotransferase II (NPTII) Protein. *Bio/Technology* 11:1543-1547.
- Fuchs, R.L., D.B. Re, S.G. Rogers, B.G. Hammond and S.R. Padgett. 1994. Safety Evaluation of Glyphosate-Tolerant Soybeans. *Proceedings from the OECD*

Workshop on Safety Evaluation of Foods, Environmental Health and Safety Division, OECD, Paris (In Press).

Hare, J.D. 1980. Impact of Defoliation by Colorado Potato Beetle on Potato Yields. *J. Econ. Entomol.* 73:369-373.

Jones, D.D. and J.H. Maryanski. 1991. Safety Considerations in the Evaluation of Transgenic Plants for Human Foods. In *Risk Assessment in Genetic Engineering*. M.A. Levin and H.S. Strauss, eds. McGraw-Hill, New York. pp 64-82.

MacIntosh, S.C., T.B. Stone, S.R. Sims, P.L. Hunst, J.T. Greenplate, P.G. Marrone, F.J. Perlak, D.A. Fischhoff and R.L. Fuchs. 1990. Specificity and Efficacy of Purified *Bacillus thuringiensis* Proteins against Agronomically Important Insects. *J. Invert. Path.* 56:258-266.

Nap, J.P., J. Bijvoet and W.J. Stikema. 1992. Biosafety of Kanamycin-Resistant Transgenic Plants: An Overview. *Transgenic Crops*. 1:239-249.

National Potato Council. 1992. *Potato Statistical Yearbook*. Englewood, CO.

Newell, C., R. Rozman, M. Hinchee, E. Lawson, L. Haley, P. Sanders, W. Kaniewski, N. Tumer, R. Horsch and R. Fraley. 1991. *Agrobacterium* Mediated Transformation of *Solanum tuberosum* L. cv. 'Russett Burbank'. *Plant Cell Reports*. 10:30-34.

Organization for Economic Co-operation and Development. 1992. *Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles*. OECD, Paris, France.

Padgett, S.R., D.B. Re, G.F. Barry, D.A. Eichholtz, X. Delannay, R.L. Fuchs, G.M. Kishore and R.T. Fraley. 1995. New Weed Control Opportunities: Development of Soybeans with Roundup® tolerance. In *Herbicide-Resistant Crops: Agricultural Economic, Environmental, Regulatory, and Technological Aspects*. S.O. Duke, editor. CRC Press (In Press).

Pariza, M.W. and E.M. Foster. 1983. Determining the Safety of Enzymes Used in Food Processing. *J. Food Protection* 46:453-468.

Perlak, F., T.B. Stone, Y.M. Muskopf, L.J. Petersen, G.B. Parker, S.A. McPherson, J. Wyman, S. Love, D. Beaver, G. Reed and D. Fischhoff. 1993. Genetically Improved Potatoes-Protection from Damage by Colorado Potato Beetles. *Plant Mol. Biol.* 22:313-321.

Shields, E.J. and J.A. Wyman. 1984. Effect of Defoliation at Specific Growth Stages on Potato Yields. *J. Econ. Entomol.* 77:1194-1199.

Sjoblod, R.D., J.T. McClintock and R. Engler. 1992. Toxicological Considerations for Protein Components of Biological Pesticide Products. *Regulatory Toxicol. and Pharmacol.* 15:3-9.

Slaney, A.C., H.L. Robbins and L. English. 1992. Mode of Action of *Bacillus thuringiensis* Toxin CryIIIA: An Analysis of Toxicity in *Leptinotarsa decemlineata* (Say) and *Diabrotica undecimpunctata Howardi* Barber. Insect Biochem. Mol. Biol. 22:9-18.

The United States Pharmacopeia. 1990. The United States Pharmacopeial Convention, Inc., Rockville, MD. p. 1788.

Taylor, S.L. 1992. Chemistry and Detection of Food Allergens. Food Technol. 39:146-152.

Taylor, S.L., R.F. Lemanske Jr., R.K. Bush and W.W. Busse. 1987. Food Allergens: Structure and Immunologic Properties. Ann. Allergy 59:93-99.

Taylor, S.L., J.A. Nordlee and R.K. Bush. 1992. Food Allergies. In Food Safety Assessment, ACS Symposium Series 484. J.W. Finley, S.F. Robinson and D.J. Armstrong, eds. American Chemical Society, Washington, D.C.

WHO. 1993. Health Aspects of Marker Genes in Genetically Modified Plants. World Health Organization Food Safety Unit, Geneva, Switzerland, 32pp.

Table 1. Comparison of the biochemical characteristics of *B.t.t.* / NPTII and known allergenic proteins.<sup>1</sup>

Characteristic	Allergens	<i>B.t.t.</i> or NPTII
Prevalent protein in food	yes	no
Stable to digestion	yes	no
Stable to processing	yes	no
Glycosylated	yes <sup>2</sup>	no

<sup>1</sup> As described by Taylor (1992) and Taylor, *et al.* (1987, 1992)

<sup>2</sup> Typically but not absolutely

Table 2. Composition of CPB-Protected and Parental Russet Burbank Potatoes

Component	CPB-Protected <sup>1</sup>		Control <sup>1</sup>		Published Range
	Mean	Range	Mean	Range	
Solids, % tuber fresh weight	19.6	18.0 - 21.0	20.0	19.6 - 20.5	16.8 - 24.5 <sup>2</sup>
Carbohydrate g/100g tuber	16.0	15.4 - 16.5	16.0	15.7 - 16.4	13 - 17 <sup>3</sup>
Protein g/100g tuber	2.1	2.1 - 2.2	2.1	2.0 - 2.1	1.4 - 2.9 <sup>3</sup>
Vitamin C mg/100g tuber	11.4	8.7 - 13.6	11.6	11.0 - 12.3	10.3 - 22.0 <sup>2</sup>
Vitamin B6 µg/100g tuber	97.2	75.4 - 119	97.2	89.2 - 105	140 - 280 <sup>3</sup>
Folic acid µg/100g tuber	6.7	5.7 - 7.7	7.0	5.2 - 8.7	4.0 - 20 <sup>3</sup>
Potassium mg/100g tuber	420	388 - 453	416	393 - 438	340 - 600 <sup>3</sup>
Glycoalkaloids mg/100g tuber	3.8	2.7 - 5.8	3.1	2.7 - 3.5	3.1 - 16.1 <sup>2</sup>

<sup>1</sup> Values are the mean of tubers obtained from seven CPB-protected or parental Russet Burbank control lines grown at two field locations. At each field location, plots for each CPB-protected line were replicated six times.

<sup>2</sup> Taken from Russet Burbank tubers grown in Aberdeen, ID (Pavek, J. *et al.*, Western Regional Variety Trial Report. 1980-1992, WRCC-27, University of Idaho, ID).

<sup>3</sup> Range of values for white potatoes, Scherz, H. and Senser, F. In Food Composition and Nutrition Tables 1989/90, Deutsche Forschungsanstalt für Lebensmittelchemie, Garching b. München, Eds Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1989, pp 542-544.

Table 3. Simulated mammalian digestive fate data for eight different proteins establish the rapid degradation of the proteins introduced into genetically-modified plants.

Protein <sup>1</sup>	Half-life <sup>2</sup>
<i>B.t.k.</i> HD-73	< 30 seconds
<i>B.t.k.</i> HD-1	< 20 seconds
<i>B.t.t.</i>	< 30 seconds
NPTII	< 10 seconds
CP4 EPSPS	< 15 seconds
GOX	< 20 seconds
ACC Deaminase	< 15 seconds
GUS	< 15 seconds

<sup>1</sup> Abbreviations - *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*); *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*); Neomycin phosphotransferase II (NPTII); 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS); Glyphosate oxidoreductase (GOX); 1-aminocyclopropane-carboxylic acid (ACC); Glucuronidase (GUS)

<sup>2</sup> Performed as described by Fuchs *et al.* (1993b) for the simulated mammalian gastric digestion.

Table 4. Mouse acute gavage studies conducted with eight different proteins confirm the food safety of these proteins that have been introduced into genetically-modified plants.

Protein	Crop(s)	Dose (mg/kg)
<i>B.t.k.</i> HD-73	Cotton	~4200
NPTII	Cotton, Potato, Tomato	~5000
<i>B.t.t.</i>	Potato	~5200
<i>B.t.k.</i> HD-1	Corn	~4000
CP4 EPSPS	Soybean, Cotton, Canola	572
GUS	Soybean <sup>1</sup>	100
GOX	Canola	~100
ACC Deaminase	Tomato	602

<sup>1</sup> GUS is not present in the soybean line targeted for commercial introduction, but was present in other soybean lines.

Table 5. Analyses of more than 450 components for 20 independent lines representing six different products have demonstrated the substantial equivalence of the products produced from genetically-modified plants.

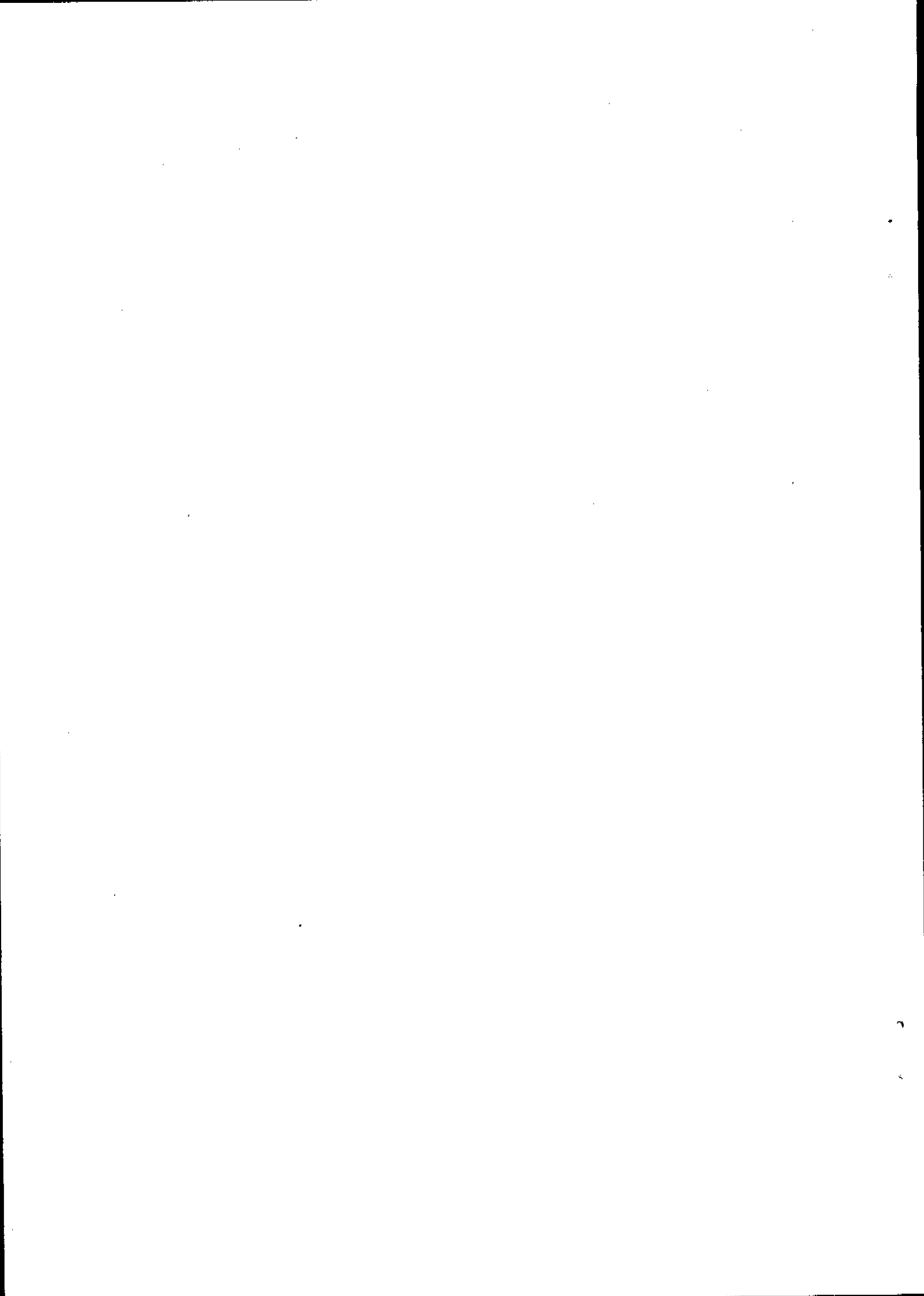
Crop/Product	Line	Components
CPB Potato	7	35
Bt Cotton	5	67
RR Canola	2	156
RR Soybean	2	80
RR Cotton	2	50
Del Rip Tomato	2	65
Total	20	453

ANNEX 7

GLOSSARY

This glossary is not intended to provide precise scientific definitions but to explain how the following terms are used within the context of this report which deals only with plants.

Conventional counterpart:	An existing acceptable food or food component which is sufficiently close in terms of composition, structure, function and use to the new food component so as to provide a safety standard against which to assess the new food component.
Critical nutrients/toxicants:	Chemical compounds present in a food or food component which are known to have nutritional or toxicological effects in humans and which, if present in the new food at levels which are significantly different from those in traditional foods or food components, could be expected to have consequences for human health bearing in mind the likely patterns of consumption of the food or food component.
Edible cultivar:	Those cultivars of a particular plant species which are used as human food or as a source of human food or food components.
Food component:	A constituent fraction of a food which is capable of being identified and characterised and may include major or minor nutrients and natural toxicants as well as macro components such as oil, protein or starch.
Gene product:	A RNA or a protein (e.g. an enzyme) the production of which, in a living plant, is directed by the corresponding gene.
Inserted gene:	A piece of DNA which has been inserted into a plant using recombinant DNA technology and which contains sufficient heritable information to direct the production of a particular gene product in that living plant.
Modern biotechnology:	Techniques, based on molecular biology, for making specific modifications to the genome of plants which have been made possible by scientific advances in the understanding of the nature and function of DNA and in the use of recombinant DNA technology.



<b>Molecular characterisation:</b>	Includes DNA sequence data and the mapping of particular functions on the plant chromosome and on the inserted DNA.
<b>Pleiotrophic effects:</b>	A phenomenon in which a single genetic alteration affects multiple phenotypic characteristics (e.g. a change in a metabolic pathway which affects multiple end products of that pathway or metabolic effects of a new gene product on the overall behaviour of a modified plant).
<b>Reference characteristics:</b>	Parameters, which may be agronomic, molecular or chemical, which are useful in making the comparison of a new food with its conventional counterpart.
<b>Substantial equivalence:</b>	A concept which embodies the idea that existing organisms used as food, or as a source of food, can be used as the basis for comparison when assessing the safety for human consumption of a food or food component which is new or is modified.
<b>Wholesomeness:</b>	The property of being favourable to health and which embraces both the nutritional and toxicological aspects of a food under the anticipated patterns of consumption.