FOOD DERIVED FROM INSECT-PROTECTED, HERBICIDE-TOLERANT CORN Bt-11

A Safety Assessment

TECHNICAL REPORT SERIES NO. 10

AUSTRALIA NEW ZEALAND FOOD AUTHORITY
November 2001

ISBN 0 642 34549 X
ISSN 1446-4977
Published November 2001

ANZFA Australia
PO Box 7186
Canberra BC ACT 2610
Australia
Tel +61 2 6271 2241
Fax +61 2 6271 2278
Email info@anzfa.gov.au

ANZFA New Zealand
PO Box 10599
Wellington
New Zealand
Tel + 64 4 473 9942
Fax +64 4 473 9855
Mail nz.reception@anzfa.gov.au

TABLE OF CONTENTS

SUMMARY

Food derived from Bt-11 corn has been evaluated to determine its suitability for human consumption. The evaluation criteria included analysis of changes at the DNA, protein and whole food levels, and assessment of the potential allergenicity and toxicity of any newly expressed proteins. Examination of these criteria has enabled both the intended and unintended changes to be identified, characterised and evaluated for safety.

Nature of the genetic modification

A proprietary inbred corn line, H8540, was transformed with two genes — the *pat* and *cry*1A(b) genes to generate Bt-11 corn. Bt-11 corn contains a single copy of each gene at one chromosomal location in the corn genome. No other genes were transferred.

The *cry*1A(b) gene is one of several genes from the bacteria *Bacillus thuringiensis*, which encode toxins collectively known as the *Bt* toxins. These toxins are selectively active against groups of insects such as moths and butterflies, beetles, and flies and mosquitos. The *Bt* toxin produced by the *cry*1A(b) gene is known as the Cry1A(b) protein and is selectively active against lepidopteran insects. This gene has been transferred to corn to protect it specifically against the European corn borer.

The *pat* gene is derived from the bacteria *Streptomyces viridochromogenes* and encodes for the enzyme phosphinothricin acetyl transferase (PAT), which enables plants to detoxify the broad-spectrum herbicide phosphinothricin (which is the active moiety of glufosinate ammonium). This protein enables the selection of genetically modified plant cells from unmodified cells and also confers herbicide tolerance to the genetically modified corn line.

The transformed corn was shown to be phenotypically and genotypically stable by segregation and mapping studies over multiple generations.

General safety issues

Corn represents a staple food for a significant proportion of the world's population. Corn-based products are routinely used in an enormous number and diverse range of foods, and have a long history of safe use. Products derived from Bt-11 corn may include highly processed corn products such as flour, breakfast cereals, high fructose corn syrup and other starch products as well as products derived from fresh sweet corn varieties (frozen, canned and powdered products).

The transformed corn produces two new proteins: Cry1A(b) and phosphinothricin acetyltransferase (PAT). The expression of both proteins in the corn kernels is low – Cry1A(b) was expressed to a maximum level of 3.17 µg/g fresh weight of sweet corn and a maximum of 1.6 µg/g fresh weight of maize varieties and the PAT protein was below the limit of detection in all lines tested. Cry1A(b) was below the level of detection in canned sweet corn.

One of the important issues to consider in relation to genetically modified foods is the impact on human health from potential transfer of novel genetic material to cells in the human digestive tract. This concern primarily refers to the presence of antibiotic resistance genes in genetically modified foods. Bt-11 corn does not contain any antibiotic resistance genes and

therefore it was not necessary to address this issue in this assessment. Transfer of the *cry*1A(b) and *pat* genes from Bt-11 corn to human cells via the digestive tract was considered to be unlikely. As the amount of novel genetic material in Bt-11 corn is minute compared to the total amount of DNA present, it is unlikely to pose any additional risks compared with the large amount of DNA naturally present in all foods.

Toxicological issues

Corn does not have any naturally-occurring toxins or allergens and has a long history of safe use.

The Cry1A(b) and PAT proteins are present at low levels in kernels of Bt-11 corn lines tested. The potential toxicity and allergenicity of the Cry1A(b) and PAT proteins were investigated.

In acute toxicity studies of the Cry1A(b) and PAT proteins in mice, there were no signs of toxicity at a dose of approximately 3.5 g/kg and 2.6 g/kg bodyweight respectively. The newly expressed proteins were readily degradable in simulated gastric conditions and neither protein has similarity with known toxins or allergens. The Cry1A(b) protein is present in low levels in kernels of both maize and sweet corn varieties, and could not be detected after processing (canning) of sweet corn. The PAT protein was below the level of detection in kernels of all varieties tested. These results suggest that dietary exposure to Cry1A(b) and PAT from consumption of Bt-11 corn kernels would be very low.

Nutritional issues

Detailed compositional analyses were assessed to establish the nutritional adequacy of Bt-11 corn and to compare it to non-modified control lines of a similar genetic background. No consistent differences in major components or nutrients were observed in Bt-11 corn varieties compared to their respective control lines, or in plants treated with herbicide compared to untreated controls.

Although some statistically significant differences were observed, these were small and random and are not considered to have any biological significance or raise any safety or nutritional concerns. All values reported in the study are consistent with ranges cited in the published literature. The results support the conclusion that Bt-11 corn is nutritionally and compositionally comparable to non-modified corn hybrids and that no health risks are associated with consumption of food derived from the genetically modified corn.

Conclusion

No public health and safety concerns have been identified in the assessment of insect protected, herbicide tolerant Bt-11 corn. Based on currently available data, the food derived from genetically modified Bt-11 corn is comparable to food derived from other commercially available corn in terms of its safety and nutritional adequacy.

FOOD DERIVED FROM INSECT-PROTECTED, HERBICIDE-TOLERANT CORN Bt-11

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on corn that has been genetically modified to, firstly, provide protection against insects, specifically the European corn borer (ECB), and secondly, to be tolerant to the herbicide glufosinate ammonium. The corn is referred to as 'Bt-11 corn'. Protection against the European corn borer is achieved through the expression in the plant of a modified, truncated version of the *cry*1A(b) gene which produces a nature identical insecticidal protein, Cry1A(b). Cry1A(b) is produced naturally by the spore-forming soil bacterium *Bacillus thuringiensis kurstaki* strain HD-1 (*B.t.k.*).

Tolerance to the herbicide glufosinate ammonium is achieved through the expression of the *pat* gene, which produces the enzyme, phosphinothricin acetyl transferase (PAT) that chemically modifies the herbicide, thus rendering it inactive.

Bt-11 corn has been crossed into both maize and sweet corn varieties. Maize varieties are generally classified into flint, pop, dent and flour lines based on the hardness of the kernel. Flint varieties are preferred by dry millers for flour, grits and meal based products such as cereals and dent varieties are preferred by wet millers for starch and starch based products such as high fructose corn syrup. Corn oil may be produced from the germ of all varieties. Fermentation of cereal grains is also used for beverage and alcohol production.

A wide variety of food products are derived from the genetically modified corn including highly processed corn-based food ingredients such as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Maize starch is also used by the food industry for the manufacture of dessert mixes and canned foods. Corn-based ingredients can also be processed into breakfast cereals, baking products, extruded confectionary and corn chips.

As well as these highly processed foods, foods produced from sweet corn varieties may be consumed as fresh, canned or frozen corn or dehydrated in powder form.

DESCRIPTION OF THE GENETIC MODIFICATION

Methods used in the genetic modification

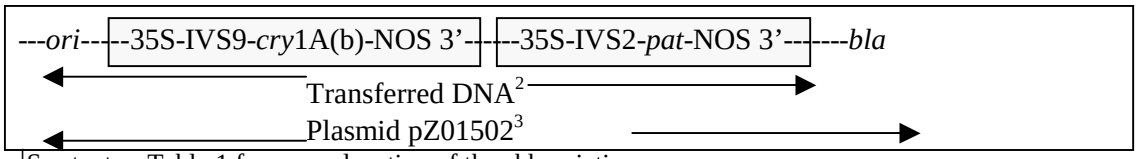
A proprietary inbred corn line, H8540, was transformed with the vector pZ01502 to transfer two new genes, a truncated *cry*1A(b) gene (referred to as the *cry*1A(b) gene) and the *pat* gene. The line was transformed using a protoplast transformation/ regeneration system similar to that described by Negrutiu *et al* (1987). The vector is derived from the plasmid pUC18 and contains the following additional sequences:

- the *bla* (or *amp*) gene under the control of a bacterial promoter, encoding a β-lactamase, which confers resistance to ampicillin;

- a nonfunctional *lac Z* gene, encoding a portion of a β-galactosidase; and
- the pUC origin of replication derived from the plasmid pBR322.

This plasmid does not contain the *tra* (transfer) and *nic/bom* (nick/basis of mobility) genes required for conjugation. The *bla* gene was used as a selectable marker when the plasmid was being generated in *Escherichia coli*, but was removed before transformation of plant cells. Thus, the transformation of corn resulted in the transfer of only one *cry*1A(b) gene and one *pat* gene. The insect-protected, herbicide-tolerant corn varieties designated 'Bt-11 corn', are the subject of this application and were derived from the original transformant.

Figure 1: Schematic diagram of pZ01502[1]



[1]See text or Table 1 for an explanation of the abbreviations.
[2]The transferred DNA is denoted by the arrows with the two boxed regions denoting the gene cassettes.
[3]The genes in the entire plasmid including the antibiotic resistance gene, *bla*.

Function and regulation of the novel genes

The genes transferred to the corn genome and their regulatory sequences are outlined in Table 1.

Table 1: Description of Genes transferred to Corn

Genetic Element	Origin	Role
***cry*1A(b)**	Bt gene from *Bacillus thuringiensis*	A crystal protein toxic to Lepidopterans
35S Promoter	cauliflower mosaic virus 35S gene	Promoter of high level constitutive gene expression in plant tissues
IVS9 Enhancer	intron from corn alcohol dehydrogenase 1S gene	A regulatory sequence that enhances gene expression in the plant
NOS 3' Untranslated region	*A. tumefaciens* nopaline synthase gene	Contains the signal for the termination of transcription and directs polyadenylation
Pat	Phosphinothricin acetyl transferase from *Streptomyces viridochromogenes*	Confers tolerance to glufosinate ammonium
35S Promoter	modified figwort mosaic virus 35S promoter	Promoter of high level constitutive gene expression in plant tissues
IVS2Enhancer	intron from corn alcohol dehydrogenase 1S gene	A regulatory sequence that enhances gene expression in the plant
NOS 3' Untranslated region	*Agrobacterium tumefaciens* nopaline synthase gene	Contains the signal for the termination of transcription and directs polyadenylation

The cry1A(b) gene

The *cry*1A(b) gene derived from the soil bacterium *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*) strain HD1 confers protection against attack from certain species of lepidoptera, including the European corn borer (ECB) (Geiser *et al*, 1986). The DNA sequence of the gene has been truncated at the 3' end and modified to increase the level of expression in corn, but the amino acid sequence of the protein has not been altered (Perlak *et al*, 1991). The

*cry*1A(b) gene in Bt-11 corn codes for the Cry1A(b) protein, a truncated version of the δ-endotoxin produced by *B. thuringiensis*.

Plasmid pZ01502 contains one copy of the *cry*1A(b) gene, controlled by the untranslated 35S promoter from cauliflower mosaic virus (CaMV) and the NOS 3' untranslated region from the nopaline synthetase gene of *Agrobacterium tumefaciens* (NOS 3'). The *cry*1A(b) gene is fused to an intron from the corn alcohol dehydrogenase 1S gene (IVS9) to enhance gene expression in the plant (Mascarenhas *et al*, 1990).

The pat gene

The *pat* gene is derived from the soil microorganism *Streptomyces viridochromogenes* strain Tu494. It codes for the enzyme phosphinothricin acetyl transferase (PAT) which modifies and inactivates the herbicide glufosinate ammonium (Strauch *et al*, 1988).

Plasmid pZ01502 contains one copy of the *pat* gene, which uses the same promoter and 3' untranslated sequence to direct initiation and termination of transcription of the mRNA as the *cry*1A(b) gene (the CaMV 35S promoter and NOS 3' termination signal). The *pat* gene is also fused to an intron from the corn alcohol dehydrogenase 1S gene (IVS2) to enhance gene expression in the plant (Mascarenhas *et al*, 1990). The native DNA sequence of the gene has been altered to optimise expression in plants (Wohlleben *et al*, 1988) but the amino acid sequence of the PAT protein is unaltered. The changes to the DNA sequence alter codon usage to lower the GC content.

The bla gene

A *bla* gene was used as a selectable marker to distinguish transformed bacterial cells from non-transformed cells. It codes for a β-lactamase enzyme that confers resistance to some β-lactam antibiotics, including the moderate-spectrum penicillin and ampicillin. Bacterial cells that contained the pZ01502 plasmid were selected through their resistance to ampicillin. The *bla* gene was excised from the gene construct before transformation of corn embryos and is therefore not present in Bt-11 corn. This has been demonstrated by Southern blot and specific-primer PCR analyses.

Characterisation of the genes in the plant

Syngenta Seeds submitted the following study regarding characterisation of the novel genes in Bt-11 and stability of genetic changes.

Hilleshög NK (1996). Molecular characterisation of the genetically modified (Bt-11) maize.

Southern blot experiments confirmed the presence of the *cry*1A(b) and *pat* genes in Bt-11 corn lines and the absence of the *bla* gene. Prior to transformation, the plasmid DNA was digested with restriction enzymes to produce the DNA fragment containing only the *cry*1A(b) and *pat* genes. The *bla* gene was specifically removed by this digest therefore producing a DNA fragment without any antibiotic resistance genes (illustrated in figure 1).

Southern blot and polymerase chain reaction (PCR) analyses of the Bt-11 corn line was used to support the absence of the *bla* gene. No positive signal was obtained when using a *bla* probe in Southern blots. PCR analysis of the genetically modified corn line, Bt-11, also

indicated that it did not contain the *bla* gene. Both Southern blotting and PCR are sensitive enough to detect a single copy of the *bla* gene.

The PCR-walking technique was used to determine that a 1.4 Kb DNA fragment of the vector sequence, upstream from the *cry*1A(b) gene including the origin of replication is transferred to the Bt-11 corn genome. The DNA fragment transferred to Bt-11 corn includes the two novel genes and the bacterial origin of replication (*ori*) from the pUC18 plasmid.

Stability of the genetic changes

The stability of the inserted DNA in Bt-11 corn was demonstrated by a Mendelian inheritance pattern. The segregation of the *cry*1A(b) and *pat* genes and their phenotypic traits was followed over multiple generations. F1 plants (first generation hybrids) were identified as containing the *cry*1A(b) and *pat* genes. These plants were self-fertilised to produce the S1 population. This S1 population was screened for protection against the European corn borer and for tolerance to glufosinate ammonium. The S1 plants were again self-fertilised. The insect protection and herbicide tolerance traits were then backcrossed into two genetic backgrounds (H8540 and 977), and in some cases, followed by further self-fertilisation.

Seed was collected from corn plants exhibiting both new traits representing different backcross stages and planted in the field for analysis in 1994 and 1995. Plants were tested for protection against the European corn borer and tolerance to glufosinate ammonium. All plants were either both tolerant to the herbicide and protected against insect attack or susceptible to both with segregation patterns consistent with the expected ratio for a single dominant locus, for that particular generation.

The stability of the insert and specifically the *pat* and *cry*1A(b) genes was also demonstrated from R_3 and R_6 generations using Southern blot analysis. Segregation analyses for Bt 11 corn for the six generations of backcrosses and also for crosses with two inbred corn lines are consistent with a stable, single dominant gene segregating according to Mendelian genetics.

Plants screened for protection against insect attack (bioassays with the European corn borer) and for tolerance to the herbicide glufosinate ammonium demonstrated these phenotypes and inheritance patterns consistently over multiple generations. These studies also demonstrated that the *cry*1A(b) and *pat* genes are closely linked, as they always segregated together.

Restriction fragment length polymorphism (RFLP) mapping was used to determine the location of the novel genes in Bt-11. The progeny of Bt-11 plants crossed with the two inbred corn lines were screened with RFLP probes, corresponding to different regions of the corn genome. Comparison of the genotypes of the progeny with isogenic controls demonstrated that the site of integration for the genetic material in Bt-11 corn is located on the long arm of chromosome 8.

Conclusion regarding the nature of the genetic modification

A single copy of the *cry*1A(b) and *pat* genes are transferred to corn resulting in the development of an insect protected (lepidopteran), herbicide tolerant (glufosinate ammonium) Bt-11 corn. Segregation analyses indicate that the transferred DNA is integrated into the corn genome as a single and stable insert. Further molecular studies indicated that the insertion site is on the long arm of maize chromosome 8.

GENERAL SAFETY ISSUES

The Bt-11 corn has been assessed according to the safety assessment guidelines developed by ANZFA, relating to Group D foods - food ingredients, i.e. plants or animals that contain new or altered genetic material (ANZFA 1999).

History of use

Corn has been cultivated for centuries and is used as a basic food item by people throughout the world (Wright, 1987). Most corn production is used for human consumption, and a wide variety of food products are derived from corn kernels. Sweet corn varieties are grown largely for human consumption. Corn grain is also widely used as an animal feedstuff.

Two milling procedures are used in corn processing – dry and wet milling. Dry milling is a mechanical process in which the endosperm is separated from the other components of the kernels and fractionated into coarse particles (grits). The process is used to produce meal and flour for use in cereals, snack foods and bakery products, or for use in brewing (Alexander, 1987). Food products derived from dry milling include flakes and grits. Corn flakes are produced by a process that involves high temperature and pressure and grits are prepared by boiling.

The wet milling process is designed to physically separate the major component parts of the kernel: starch, protein, oil and fibre. Wet milling produces primarily starch (typically 99.5% pure). In this process grain is steeped in slightly acidic water for 24–48 hours before being milled. Starch is separated from other solids through a number of grinding, washing and sieving steps. Washed starch may contain 0.3-0.35% total protein and 0.01% soluble protein. These treatments would be expected to degrade and remove proteins (May, 1987). Oil is produced from wet-milled corn by solvent extraction and heat (i.e. 120ºC) and corn oil is considered free of protein (Rogers, 1990).

Bt-11 has been crossed with elite maize and sweet corn hybrid varieties. Grain harvested from Bt-11 maize corn (i.e.. predominantly dent corn varieties) will be consumed only after processing as either starch based products like high fructose corn syrup or dry milling corn-based products such as breakfast cereals and flour. Bt-11 sweet corn may also be consumed fresh, canned, frozen or dehydrated in powder.

A summary of the Bt-11 lines analysed is given in Table 2. These have been divided into the elite dent and sweet corn hybrid lines. Additionally, compositional data for genetically modified plants that have been treated with herbicide during growing have been analysed.

Table 2. Summary of lines evaluated in the application[1].

Lines	Protein Expression	Proximate[2]	Fatty Acids	Amino Acids[3]	Vitamins & Minerals
Initial transformant (greenhouse data)					
H8540 Bt$^+$/Bt$^+$		+	+	+	
Control H8540		+	+	+	
H8540 Bt$^+$/Bt$^-$ hybrid		+	+	+	
Control hybrid		+	+	+	
Dent Corn					
N4640-CBR					+
X4734-CBR	+	+	+	+	
X4334-CBR	+	+			
N4242-CBR					+
N4640		+	+	+	+
NK4242	+	+			+
X6534-CBR	+	+	+	+	
X6514		+			
N6800			+	+	
X7634-CBR	+	+			
X7514	+	+			
Sweet Corn Varieties					
0943	+	+			+
Jubilee	+	+			+
0937	+	+			+
Bonus	+	+			+
0941	+	+			+
Empire	+	+			+
Herbicide treated plants					
Madera-Bt		+			+
Madera		+			+
Manuel-Bt		+			+
Manuel		+			+
Magister-Bt		+			+
Magister		+			+

[1]A "+" indicates the data that was provided for that line. Control lines are in italics and genetically modified corn lines are in bold and are denoted as CBR – corn borer resistant or Bt. Control lines are either corresponding isogenic non-GM lines or are of a similar genetic background.

[2]Proximate components analysed were: *Initial transformants*: Total nitrogen, moisture, ash, starch, cellulose, xanthophyll; *Dent corn*: protein, oil, starch and fibre; *Sweetcorn*: moisture, protein, fat, ash, carbohydrates (total), calories, calories from fat, sugars, other carbohydrates, total dietary fibre; *Treated*: energy, carbohydrate, protein, fat, fibre.

[3]Some analyses did not assess all amino acids.

Corn-based food products are derived from many different corn varieties, particularly dent corn lines and sweet corn lines. The applicant has provided data on the original transformant (H8540 and hybrids), and has extended their analysis to those Bt-11 corn lines that are widely used in food production. This includes several dent corn and sweet corn lines that have been developed from conventional breeding with the original transformed line. This information on additional lines enables a comprehensive analysis of the potential impact of the novel genes in different corn genotypes.

Nature of novel proteins

Two new proteins are expressed in Bt-11 corn: a truncated form of the insecticidal protein Cry1A(b), and phosphinothricin acetyl transferase (PAT). The protein products of the novel genes in the transgenic corn have been characterised and the extent of expression determined.

Cry1A(b)

The *cry*1A(b) gene transferred to Bt-11 corn codes for the Cry1A(b) protein, which is an identical but truncated version of the δ-endotoxin produced by *B. thuringiensis*. In the gut of a susceptible insect, the δ-endotoxin is broken down to yield a smaller protein that binds to specific receptors and lyses cells in the gut, preventing feeding and thus causing death.

During sporulation, *B. thuringiensis* produces cytoplasmic inclusions containing one or more of the insecticidal crystal proteins. Most crystal proteins are synthesised intracellularly as inactive protoxins that spontaneously form small crystals, approximately 1 µm in size. Upon ingestion by susceptible insects, the highly alkaline pH of the midgut promotes solubilisation of the protoxin-containing crystals. The protoxin is then activated by trypsin-like gut proteases which cleave off domains from the carboxy- and amino- termini, leaving a protease resistant core which is the active toxin. The active toxin binds to a highly specific glycoprotein receptor on the surface of midgut epithelial cells in the insect. Aggregation of the core toxins results in the formation of a pore through the cell membrane. These cells eventually swell and burst` causing loss of gut integrity and resulting in larval death within 1 to 2 days (Hofte and Whitely, 1989; Schnepf et al, 1998)

The Cry1A(b) protein produced by *B. thuringiensis* subsp. *kurstaki* is a 130 kDa protoxin, which is cleaved in the gut of a susceptible insect to give an insecticidally active 65 kDa fragment. This fragment can be generated *in vitro* by digestion of the protoxin with trypsin. The modified truncated *cry*1A(b) gene product in Bt-11 corn is a protein of 615 amino acids identical to the first 615 amino acids of the native protein, with a molecular weight of approximately 65 kDa.

PAT

S. viridochromogenes produces the tripeptide antibiotic, bialaphos, which consists of phosphinothricin, an analogue of L-glutamic acid bearing two alanine residues. Peptidases hydrolyse bialophos releasing free phosphinothricin. The *pat* gene encodes phosphinothricin acetyl transferase (PAT) which breaks down bialaphos thus allowing the microorganism to protect itself against the toxic compound it produces. When transferred to plants, the *pat* gene product enables the plant to detoxify the broad-spectrum herbicide phosphinothricin (the active moiety of glufosinate ammonium herbicides).

In plants, the enzyme glutamine synthetase, plays a central role in the uptake of nitrogen by catalysing the incorporation of ammonia into glutamine. The herbicide glufosinate ammonium inhibits this enzyme in plants, leading to an accumulation of ammonia in the tissues, which kills the plant. The PAT protein catalyses the acetylation of phosphinothricin, eliminating its herbicidal activity. Acetylation of phosphinothricin produces N-acetyl-glufosinate (NAG) and two further metabolites, 3-methylphosphinicopropionic acid (MPP) and 3-methylphosphinicoacetic acid (MPA).

Although Bt-11 corn is marketed only as an insect-protected plant, the presence and expression of the *pat* gene enables tolerance to commercial applications of the herbicide glufosinate ammonium and is therefore also regarded as a herbicide tolerant plant. The expression level of the PAT protein is discussed in detail in section 3.3. Bialophos, an antibiotic produced by *S. viridochromogenes* is the natural substrate for PAT. No additional substrates, apart from phosphinothricin, have been reported.

Expression of novel protein in the plant

Studies evaluated:

Schramm S. and Warnick D. (1998). Quantification of Cry1A(b)protein in Attribute insect-protected sweet corn tissues, whole plants and processed products. Performing laboratory: Novartis Seeds Inc, Gilroy, CA, USA. Determination of phosphinothricin N-acetyl-transferase levels in Bt11 maize. Performing laboratory: Xenos Laboratories Inc.

The expression of the PAT and Cry1A(b) proteins in Bt-11 plants has been determined for several maize lines grown both in field trials and in greenhouses and also for three sweet corn lines (refer to Table 2). Expression levels of the introduced proteins were measured using enzyme linked immuno-sorbent assay (ELISA), which is a highly sensitive technique that can detect the presence of a protein generally to a sensitivity of 10 - 100 pg.

In a greenhouse experiment, various plant tissues at several stages of development were analysed for the novel proteins. A second experiment determined the expression levels for four Bt-11 maize hybrids grown in two locations (i.e. 2 hybrid lines per location) and a third study determined the level of the novel proteins in three sweet corn hybrids. ELISA analysis was used in the analysis of leaf tissue, kernel and canned kernels from the Bt-11 corn.

ELISA analysis of the Cry1A(b) protein levels in Bt-11 corn plants grown in the greenhouse determined that the highest levels were found in the leaf tissue (Table 3) with the highest level at day 25 on the fifth leaf (data not shown).

Table 3: Specific concentration of the Cry1A(b) protein in Bt-11 dent corn tissues during the life cycle of plants grown in the greenhouse[1].

	ng Cry1A(b)/mg plant protein – days post planting (± SE)				
Tissue	**10**	**25**	**59**	**84**	**119**
Roots	11.7 ± 1.7	-	12 ± 3.4	18.2 ± 4	2.2 ± 1.2
2nd Leaf	106 ± 4.7	125 ± 5	-	-	-
15th Leaf	-	-	37.9 ± 2.2	10.2 ± 1.1	-
Pollen	-	-	1.25 ± 0.8	-	-
Kernel	-	-	-	8.2 ± 2.5	0.4 ± 0.4

[1]Values are means of samples from 5 replicate plants (n=5). Data points that are not available at a certain developmental stage are denoted as '-'.

The Cry1A(b) protein was detected in all plant tissue samples. A summary of the results from the greenhouse tissues is given in Table 3. Generally higher levels were detected at the younger stages of tissue development. The level of Cry1A(b) protein decreased as the plant reached full maturity and the tissues became senescent.

A second analysis was done on leaf, husk, stalk and kernels for four Bt-11 corn hybrids grown in field trials and respective control corn lines that have similar background genetics. All tissues were physiologically mature, green and healthy when sampled: leaf - distal half of the ear and next leaf up; stalk: 20 cm section from the stalk above ear; husk: the upper third of the outer husk leaf. The kernels from one location were picked at the early dent stage and at the late dent stage at the second location. The Cry1A(b) protein is expressed at very low levels in these tissues (Table 4). This is equivalent to less than 0.02% of the total protein in the seed. The highest level of the Cry1A(b) protein was found in leaf tissue, with the other plant tissues having significantly lower levels of the protein. The four hybrids produced similar levels of the Cry1A(b) protein.

The PAT protein was analysed in two Bt-11 hybrid corn lines. The protein level is below the limit of detection (i.e. 1ng/ml extract) in the kernel, husk and stalk and is expressed in trace amounts in the leaf (Table 4). The level of the PAT protein in the leaf represents less than 0.0005% of the total protein.

Table 4: Mean levels of the Cry1A(b) and PAT proteins in corn tissues[1].

		Mean levels in leaf and kernel (µg/g fresh weight)			
		leaf	**kernel**	**husk**	**stalk**
X4334-CBR	Cry1A(b)	4.3 ± 0.66	1.5 ± 0.21	1.1 ± 0.26	0.71 ± 0.11
	PAT	0.0386 ± 0.0029	lod[2]	lod	lod
X4734-CBR	Cry1A(b)	5.05 ± 0.35	1.30 ± 0.28	0.84 ± 0.18	0.55 ± 0.06
	PAT	0.0494 ± 0.005	lod	nd	nd
Control NK4242	PAT	lod	lod	lod	lod
X6534-CBR	Cry1A(b)	5.30 ± 0.90	1.50 ± 0.04	0.79 ± 0.03	0.64 ± 0.04
X7634-CBR	Cry1A(b)	5.24 ± 0.78	1.60 ± 0.13	1.04 ± 0.23	0.53 ± 0.06
Control NK7514	Cry1A(b)	0	0	0	0

[1]n=4 for all Cry1A(b) means and n=3 for all PAT means.
[2]lod (limit of detection) for the procedure is 1ng PAT/ml extract. These values are considered not above background. nd: no data

A third analysis determined the level of the Cry1A(b) protein in tissues from three Bt-11 sweet corn hybrid varieties and control lines with a similar genetic backgrounds (Jubilee, Bonus and Empire). The Cry1A(b) protein levels in kernels tested at prime harvest stage were also assessed in these sweet corn hybrids that had been canned.

The level of the Cry1A(b) protein was present at low levels (Table 5) in Bt-11 sweet corn hybrids. Cry1A(b) protein was not detectable in any of the canned corn samples tested.

Given the low levels of the Cry1A(b) protein determined in kernels for all Bt-11 corn varieties (field and sweet corn) and that it was not detected in canned corn, dietary exposure to the novel protein is expected to be very low (discussed under Nutritional Issues).

Table 5: Cry1A(b) protein levels in tissues from Bt-11 sweet corn hybrids[1].

	Cry1A(b) levels in Bt-11 tissues (µg/g fresh weight)					
	Leaves		Kernel		Canned[3]	
	mean	range	mean	range	mean	range
Control[2]	0	-	0	-	nd	-
Hybrid 0943	4.53	3.87-5.18	3.17	2.54-3.80	nd	-
Hybrid 0937	3.10	2.60-3.86	1.59	1.41-1.80	nd	-
Hybrid 0941	3.31	2.66-3.92	0.78	0.51-1.08	nd	-

[1]Values are µg/g fresh weight. n=3 for all means except in leaves and kernels from 0943 where n=2.
[2]Control plants varieties are Jubilee, Bonus and Empire. Control plants had ELISA values corresponding to 0ng Cry1A(b)/g fw.
[3]The absorbance generated for canned samples did not exceed background (nd = not detectable). The lower limit of quantification was 2ng/g fw

Impact on human health from potential transfer of novel genetic material to cells in the human digestive tract

The human health considerations in this regard depend on the nature of the novel genes and must be assessed on a case-by case basis.

In 1991, the World Health Organization (WHO) issued a report of a Joint FAO[1]/WHO Consultation which looked at strategies for assessing the safety of foods produced by biotechnology (WHO 1991). That consultation concluded that as DNA from all living organisms is structurally similar, the presence of transferred DNA in food products, in itself, poses no health risk to consumers.

The major concern in relation to the transfer of novel genetic material to cells in the human digestive tract is with antibiotic resistance genes. Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA *per se* (WHO 1993). There have been concerns expressed, however, that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of antibiotics.

No antibiotic resistance genes were transferred to Bt-11 corn as indicated by Southern blot and PCR analysis.

In relation to transfer of novel genetic material from genetically modified food to human cells via the digestive tract, this is also equally unlikely to occur. In considering the potential impact on human health, it is important to note that humans have always consumed large amounts of DNA as a normal component of food and there is no evidence that this consumption has had any adverse effect on human health. Furthermore, current scientific knowledge has not revealed any DNA sequences from ingested foods that have been incorporated into human DNA. Novel DNA sequences in genetically modified foods comprise only a minute fraction of the total DNA in the food (generally less than 0.01%) and are therefore unlikely to pose any additional risks compared with the large amount of DNA naturally present in all foods.

[1] Food and Agriculture Organization.

Conclusions regarding general safety issues

The *cry*1A(b) and *pat* genes are expressed at low levels in Bt-11 corn. Both proteins are expressed highest in the leaf tissue. The expression level of the Cry1A(b) protein is much lower in the kernel representing less than 0.02% total protein in the seed and the PAT protein is below the limit of detection in the kernel. The level of DNA and protein in highly processed corn based products is expected to be very low and in some cases, negligible. It is also likely that the proteins will be degraded and/or removed during processing steps.

No antibiotic resistance genes were transferred to Bt-11 corn during the transformation process. The novel genetic material in Bt-11 corn comprises only a minute fraction of the total DNA present in the corn and is therefore unlikely to pose any additional risks.

TOXICOLOGICAL ISSUES

Levels of naturally-occurring toxins

Corn contains no naturally-occurring toxins that occur at biologically significant levels (Wright, 1987).

Potential toxicity of the novel proteins

The potential for toxicity of the newly expressed proteins, Cry1A(b) and PAT, were evaluated based on:

. the amino acid sequence similarity with known toxins;
. acute toxicity testing in mice;
. the resistance to digestion by proteases and acids in the model digestive/gastric system;
. their presence as a major protein component in a specified food.

The potential for acute toxicity of the Cry1A(b) and PAT proteins was assessed by evaluating physical and chemical characteristics of the proteins and also by acute oral toxicity in mice. The scientific basis for using an acute test is that known protein toxins generally act via acute mechanisms (Jones and Maryanski 1991). Another study was submitted that demonstrated equivalence of the corn-expressed Cry1A(b) protein to the microbially produced Cry1A(b) protein in terms of molecular weight, immunological reactivity, trypsin resistance, amino acid sequence, glycosylation and bioactivity.

Studies evaluated:

Kuhn JO (1994a). Cry1A(b) B.t.k. delta-endotoxin. Acute oral toxicity study in mice. Performing Laboratory: Stillmeadow Inc, Sugar Land, Texas. Sponsor: Ciba-Geigy Corporation, Research Triangle Park, NC, USA.

Kuhn JO (1995). Phosphinothricin acetyltransferase (Sample PAT-0195) Acute oral toxicity study in mice. Performing Laboratory: Stillmeadow Inc, Sugar Land, Texas. Sponsor: Ciba-Geigy Corporation, Research Triangle Park, NC

Meeusen R. and Mettler I. (1994), revised by Goy P.A. (1998). Equivalence of plant and microbially produced Bacillus thuringiensis subsp. kurstaki HD-1 protein. Performing laboratories: Novartis Seeds/Northrup King Co, University of Wisconsin, Kendrick Laboratories and Washington University School of Medicine, USA.

Cry1A(b)

(i) History of consumption

Cry1A(b) has a long history of safe use as an insecticide and has been repeatedly shown to be non-toxic to humans and other vertebrates. There is no evidence from the history of long use that there is any associated toxicity to humans. The toxicity of this protein is very specific to Lepidopteran insects. The lack of activity against non-target species appears to be due to a number of factors including physical differences in the gut environment and an absence of Cry1A(b)-specific gut receptors in other organisms (Frick, 1995). Additionally, there is evidence that demonstrates that the mammalian gut contains receptors that are not comparable to those found in the gut of susceptible insects. *In vivo* studies with rats given Cry1A(b) orally, and *in vitro* binding studies with gut tissue isolated from rats, mice, rhesus monkeys and humans did not reveal receptors for the protein (Noteborn *et al* 1995).

(ii) Similarity with known toxins

An amino acid sequence comparison of the Cry1A(b) protein to a database of 2632 sequences detected significant similarities only to other *B. thuringiensis* insecticidal crystal proteins. The sequences were obtained from the GenBank, EMBL, Swissprot and PIR databases.

(iii) Equivalence of the plant Cry1A(b)protein to the bacterially produced protein.

The test protein used in acute toxicity tests and characterisation studies was produced in *E. coli* because the genetically modified corn plants did not express enough protein for purification of large quantities. Data was presented to indicate that the bacterially produced Cry1A(b) protein is equivalent to the plant produced Cry1A(b) protein in terms of its molecular mass, N-terminal amino acid sequence, lack of glycosylation, and biological activity. The *E. coli* produced Cry1A(b) is considered a suitable substitute for plant produced Cry1A(b) in toxicity testing.

In this study, the trypsin resistant fragment of Cry1A(b) expressed by Bt-11 corn was purified by extraction of leaf tissue, trypsin digestion and immunoaffinity purification. Analysis by SDS-polyacrylamide gel electrophoresis and Western blotting demonstrated that two related proteins are present in Bt-11 corn; one of 69 kDa (the full 615 residues coded for by the *cry*1A(b) gene) and one of 65 kDa (the expected size if the first 28 amino acids have been removed by proteolysis). Both proteins are reactive with antibodies to microbially-produced Cry1A(b). Trypsin treatment resulted in a single band of 65 kDa, which is equivalent to the trypsin resistant fragment of the native protein. Some lower molecular weight immunoreactive material (42 and 15 kDa) was also present, probably representing partially digested Cry1A(b) protein. Similar results were obtained with the microbially-produced Cry1A(b) protein.

N-terminal amino acid sequencing confirmed that the Bt-11 65 kDa protein had the expected sequence of a fragment extending from residue 29 of the native protein, consistent with the fragment having been cleaved at the trypsin sensitive site at residue 28. There was no evidence of glycosylation of either the Bt-11 or the microbially-produced CryIA(b). The plant and microbially-produced Cry1A(b) had similar bioactivity against ECB, with LD_{50}'s of 0.47 and 0.50 µg/mL respectively.

(iv) Acute oral toxicity in mice – native Cry1A(b)

Hsd:S-D ICR albino mice (source: Harlan Sprague Dawley Inc, Texas) were acclimatised for at least 5 days before dosing (5/sex). They were housed individually in controlled conditions with free access to food and water, except for the 16 hours before dosing when food was withheld. *Bacillus thuringiensis* Cry1A(b) δ-endotoxin (lot BFL0194, purity 70%, source SIGMA Chemical Co, produced in *E. coli*) in carboxymethylcellulose was administered to the mice (5/sex) at 5050 mg/kg bw by single oral gavage. A 20% w/v concentration in 2% w/v aqueous carboxymethylcellulose was used, as this was the highest concentration able to be administered through the gavage tube.

Mice were observed for clinical signs at least 3 times on the day of dosing and once daily after this for a 14-day observation period. There did not appear to be any ill effects from the dosing volume. Bodyweight was determined predosing (day 0) and on days 7 and 14. At the end of the study, mice were killed for post-mortem examination of gross pathology. Any abnormalities were recorded and the gastrointestinal tracts were preserved in formalin for later histopathological examination if required.

There were no deaths during the study. The only abnormal clinical sign observed in the test group was piloerection (hair standing on end), which occurred only on day 1. During the second week after dosing, one female in the test group lost weight; all other mice showed normal bodyweight gains for their age and sex. No abnormalities were detected on necropsy. The acute oral LD_{50} for Cry1A(b) δ-endotoxin in mice is therefore 70% of 5050 mg/kg bw (i.e. 3535 mg/kg bw given that the protein was 70% pure). These results are consistent with other studies on the acute toxicity of Cry1A(b) in mice and in rabbits (Noteborn *et al* 1995, Sanders *et al* 1998) and do not demonstrate any potential mammalian toxicity from Cry1A(b) protein.

PAT

(i) History of consumption

The *pat* gene encodes the phosphinothricin-N-acetyl transferase enzyme which has a very narrow substrate specificity for phosphinothricin and demethyl-phosphinothricin, both of which are not found in humans. Acetyl transferases are a class of enzymes common to all bacterial, plant and animal cells and play a major role in both the synthesis and oxidation of fats. Since proteins from this family are naturally present in virtually all cells, they can be considered a component of the human diet.

(ii) Similarity with known toxins

A comparison of the amino acid sequence of the PAT protein to a database of known toxins demonstrated that it does not share any significant similarity with any known protein toxins. The sequences were obtained from the GenBank, EMBL, Swissprot and PIR databases. Additionally, no reports were found of toxicity associated with acetyl transferases as a class and that the donor organism has no known pathogenic potential.

(iii) Equivalence of the plant PAT protein to the bacterially produced protein.

PAT expression was at the limit of detection in Bt-11 corn plants and it was not possible to

extract it in sufficient quantities to be used in model digestion system or oral toxicity studies or to be compared to the bacterially produced protein. The PAT protein was therefore derived from expression of the recombinant protein in *E. coli*. However, the modified *pat* gene transferred to corn plants produces a protein of 183 amino acids, the sequence of which is identical to that of the PAT protein encoded by the native *pat* gene.

Based on the *pat* gene construct, there is no reason to expect that the plant produced PAT protein would be different in any way to the bacterially produced PAT protein.

(iv) Equivalence of the PAT protein produced by the bar *gene.*

Phosphinothricin acetyl transferase is also produced by *Streptomycin hygroscopicus* (Thompson *et al*, 1987) which is encoded for by the *bar* gene. A functional and structural comparison of both protein products has concluded that both proteins have comparable molecular weights and show similar immuno-cross-reactivity to their respective polyclonal antisera (Wehrmann *et al*, 1996). Both enzymes have a similar substrate affinity (for L-phosphinothricin) and do not acetylate any of the other L-amino acids tested. Both proteins were rapidly broken down in model digestion system studies and had decreased enzymatic activity (Wehrmann *et al*, 1996). These studies are discussed in the next part and also under Section 4.4.

(v) Acute oral toxicity in mice – bacterially produced PAT

Hsd:S-D ICR albino mice (source: Harlan Sprague Dawley Inc, Texas) were housed individually in controlled conditions with free access to food and water, except for the 16 hours before dosing when food was withheld. Groups (5/sex) of mice were given a single oral dose (gavage) of PAT protein (PAT-0195, purity 51% phosphinothricin acetyltransferase, expressed by the *bar* gene in *E. coli*) in carboxymethyl cellulose; heat inactivated PAT (PAT-0195C, 52% purity) in carboxymethyl cellulose; or carboxymethyl cellulose to a total dose of protein of approximately 2600 mg/kg bw (i.e. 51-52% of 5050 mg/kg bw, given that this was the purity of the protein).

Mice were observed for clinical signs at least 3 times on the day of dosing and once daily after this for a 14-day observation period. Bodyweight was determined predosing (day 0) and on days 7 and 14. At the end of the study, mice were killed for post-mortem examination of gross pathology.

One male receiving the test substance died during the study. The only notable clinical signs were decreased activity, piloerection and ptosis (drooping eyelid) on days 6–8 in the male that died. One male receiving the reference substance showed slight piloerection on the day of dosing. However, as no other clinical signs were observed in animals of any group, these signs are not considered to be treatment related. Bodyweight gain was unaffected by treatment, except in the male that died. There were no abnormal findings on post-mortem of animals surviving until the end of the study. The results do not indicate any potential toxicity from the PAT protein.

Levels of naturally-occurring allergenic proteins

Corn does not contain any known naturally-occurring allergens (Wright 1987).

Potential allergenicity of novel proteins

Although there are no simple predictive assays available to assess the allergic potential of proteins, a number of characteristics are common among many of the allergens that have been characterised. For instance, amino acid sequence similarity with known allergens may be a useful gauge of allergenic potential. A string of 8-12 consecutive amino acid residues in common with known allergens could be an indicator for allergenicity given that many T-cell epitopes of allergenic proteins are that length (Taylor and Lehrer, 1996). In terms of the chemical and physical nature of proteins, known allergens tend to be glycosylated proteins with a molecular weight of 10–70 KDa (Lehrer *et al*, 1996). Allergens also tend to be heat stable as well as resistant to peptic and tryptic digestion and the acidic conditions of the stomach. Consequently, many allergenic factors tend to be resistant to proteolytic digestion (Taylor and Lehrer, 1996). The Cry1A(b) and PAT proteins are evaluated for potential allergenicity against these criteria: molecular size, amino acid sequence similarity to known allergens, and how easily the protein is degraded by heat, acid and gastric enzymes (Lehrer and Reese 1998, Jones and Maryanski 1991).

Syngenta Seeds submitted three studies relevant to the possible allergenicity of the novel proteins which are listed below. The *in vitro* digestibility of the proteins was investigated to consider the potential allergenicity of the novel protein products which can be related to the presence of large undigested protein molecules.

Studies evaluated:

Privalle L (1994). In vitro digestibility of Cry1A (b) protein from Bt maize (corn) and Bacillus thuringiensis subspecies kurstaki under simulated mammalian gastric conditions. Ciba Seeds. Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA.

Privalle L (1994). In vitro digestibility and inactivation of the bar marker gene product phosphinothricin acetyltransferase (PAT) under simulated mammalian gastric conditions. Ciba Seeds. Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA.

Meeusen R. and Mettler I. (1994), revised by Goy P.A. (1998). Equivalence of plant and microbially produced Bacillus thuringiensis subsp. kurstaki HD-1 protein. Performing laboratories: Novartis Seeds/Northrup King Co, University of Wisconsin, Kendrick Laboratories and Washington University School of Medicine, USA.

Cry1A(b) protein

As described in Section 3, the Cry1A(b) protein produced by Bt-11 corn was demonstrated to be equivalent to the microbially-produced protein in terms of the N-terminal sequence, immunoreactivity and post-translational modification. The microbially-produced protein is considered to be a suitable substitute for plant-expressed Cry1A(b) for allergenicity studies.

(i) Physical properties of the protein

The Cry1A(b) core protein has a molecular weight of 63 kDa, which is in the size range of known allergens.

The amino acid sequence of the Cry1A(b) protein was compared to the amino acid sequences of 219 known allergens present in public domain databases (eg GenBank, EMBL, Swissprot, PIR). No biologically similarity was found with any of these known allergens.

(ii) Model digestive system studies

Native Cry1A(b) protein obtained from *Bacillus thuringiensis* subsp *kurstaki* was digested under simulated gastric conditions. The protein was extracted from a cell paste of *Btk* strain HDI-9. Simulated gastric fluid (SGF) was prepared containing NaCl, HCl and pepsin. The pepsin content (X) was initially 3.2 mg/mL, with a pH of 1 to 1.2. Solutions of SGF containing dilutions of pepsin (0.1, 0.01 and 0.001 times the standard dilution) were also prepared to investigate the degradation of the protein over time. Gastric fluid without pepsin was also prepared.

In an initial trial, 10 µL of protein sample (100 µg of protein) was added to 90 µL of SGF. A 50 µL aliquot was immediately removed, neutralised and heated to 75^{o}C for 10 minutes. The remainder was incubated at 37^{o}C for 2 minutes before neutralising and heating. Following the initial trial, a trial to investigate the time course of degradation was performed using 0.01X pepsin solution. 40 µL of protein sample was added to 360 µL of SGF. 50 µL was removed at 0, 1, 2, 5, 10 and 30 minutes and neutralised and heated as above. The protein content of each sample (in the initial and time course trial) was analysed by western blot.

Following incubation with a solution containing a standard quantity of pepsin, the native Cry1A(b) protein was almost all degraded after 2 minutes. In the time course trial, the protein was undetectable after 5 minutes with 0.01 times the standard dilution of pepsin.

This trial using simulated gastric conditions indicates that Cry1A(b) protein obtained directly from *Bacillus thuringiensis* subsp *kurstaki* is digested as normal dietary protein, being rapidly degraded under simulated gastric conditions. This result is consistent with published studies (Noteborn *et al* 1995, Sanders *et al* 1998). As Cry1A(b) produced by Bt-11 was found to be identical to the microbially produced protein (as discussed in Section 3), it can be concluded that the Bt-11 Cry1A(b) would rapidly degrade in the digestive tract. As Cry1A(b) is present at low levels in the kernel, is easily digested and does not show any amino acid sequence similarity with known allergens, it is not considered to be allergenic.

PAT protein

(i) Physical properties of the protein

A comparison of the amino acid sequence of the PAT protein to a database of known allergens demonstrated that it does not share any significant similarity with any known protein allergens. Additionally, acetyltransferases in general have no similarity to any reported mammalian allergens.

(ii) Model digestive system studies

The PAT protein used in this trial was obtained from an *E. coli* expression system and was purified following fermentation. SGF contained NaCl (2 mg/mL), HCl and pepsin (3.2 mg/mL), the pH was 1.0 to 1.2, and the activity of the fluid was determined before use.

Solutions were prepared containing successive dilutions of pepsin (0.1, 0.01 and 0.001 times the standard dilution). The reactions were started by adding 10 µL to PAT sample (26 µg total protein) to 90 µL of the appropriate gastric solution. After mixing, 50 µL was removed, neutralised and heated. This sample was designated the time zero sample. The remainder was

incubated for 2 minutes before neutralisation and heating. The presence of PAT in the fluid following incubation was determined by SDS-PAGE analysis. The enzymic activity of the solution was also determined at the pH optimum for the enzyme, at gastric pH and following serial incubation with a gastric solution containing 0.0032 mg/mL pepsin.

In the presence of SGF containing a standard concentration of pepsin, the PAT protein was completely degraded at time zero. After 2 minutes of incubation with 0.1 or 0.01 times the standard pepsin concentration, PAT degradation appeared complete. When 0.001 times the standard concentration was used, a significant amount of PAT remained after a 2-minute incubation period. This concentration was thus selected for the enzyme inactivation studies.

The enzyme activity of PAT decreased to 56% of initial values after a 10-minute incubation at 37^{o}C. This reflects the thermal sensitivity of the enzyme above 35^{o}C, and would represent the maximum activity were gastric pH or pepsin to have no effect on PAT activity. Immediately after addition to SGF without pepsin, PAT activity decreased to 2.6% of the initial activity, and reached zero by 1 minute. When pepsin was included in the SGF, the initial activity was even lower. Activity was not restored by neutralisation, indicating that inactivation of the PAT enzyme was irreversible. The half-life of the PAT protein in SGF containing 0.0032 mg/mL was between 1 and 2 minutes.

This study demonstrates that PAT loses enzymatic activity immediately upon exposure to gastric pH, and that the protein is readily digested in the stomach. As the PAT protein is present at low levels in the kernel, is easily digested and does not show amino acid sequence similarity with known allergens, it is considered highly unlikely to be allergenic.

Conclusions regarding toxicological issues

Analysis of the physical and chemical properties of the Cry1A(b) and PAT proteins have not revealed any similarities to known toxins and allergens. No adverse reactions were observed in mice that were administered either protein in acute toxicity tests. No evidence suggests that either protein has been derived from a potentially toxic or allergenic source and the Cry1A(b) protein has a long history of safe use. Both proteins are present in corn kernels at low levels and are shown to degrade in conditions that mimic the human digestive system. Therefore it is highly unlikely that either the Cry1A(b) or PAT protein would be toxic or allergenic to humans.

NUTRITIONAL ISSUES

Nutrient analysis

The safety assessment includes an analysis of the composition of the food in comparison with other commercial varieties of the crop. Given that food is produced from many corn varieties, the applicant has provided data on several different dent and sweet corn varieties. Refer to Table 2 for a complete summary of the lines analysed.

Four major studies have been conducted on Bt-11 kernels that assess the major components in inbred and hybrid lines at different stages of maturity and a comparison with their respective near-isogenic controls. The first study is an analysis of the glasshouse grown original transformant. The second suite of studies have been conducted on six dent corn lines developed from conventional breeding with the original transformant. The third set of data

has been provided on sweet corn lines also derived from conventional breeding with the original transformant. A final study assesses the potential effect of Bt-11 corn treated with the herbicide glufosinate ammonium during growing.

Studies evaluated:

Compositional analysis of Bt11 maize: determination of the substantial equivalence — chemical composition analysis done with Bt-11 maize with a European background. Performing laboratory: Association Generale des Producteurs de Mais).

Compositional analysis of Bt11 maize: determination of the substantial equivalence — chemical composition analysis done with Bt-11 maize with a US background. Part 1: Properties of grain produced from ECB protected maize hybrids; Part 2: Characterization of grain attributes of normal, wild-type maize hybrids and the Bt11 converted iso-hybrid counterparts; Part 3: Analyses of fatty acid and amino acid profiles of grain from Bt-11 maize. Report No. NSB-004-97.Performing laboratory: Novartis Seeds/Northrup King Co.

Comparison of nutritional composition of fresh and canned grain prepared from Attribute insect protected and control sweet corn hybrids. Report No. NSV-002-98. Novartis Seeds Inc.

Comparison of vitamin and mineral composition of Bt11 maize and non-modified maize hybrids. Report No. NSB-004-97. Novartis Seeds.

Goy P.A. (1999). Novartis Seed's genetically modified Bt11 maize: biochemical composition of kernels from plants treated with a glufosinate ammonium herbicide.

Study 1: Analysis of Bt-11 corn grown in greenhouses in Europe

The following greenhouse grown plants were analysed: an inbred line (H8540-Bt), a hybrid line (hybrid Bt^+/Bt^-) and their respective controls (isogenic non-modified H8540 and control hybrid). Between 45 and 56 ears were taken from each plant. Ears were harvested and dried four months after sowing and 500 g samples were analysed for moisture, total nitrogen, ash, starch, cellulose, xanthophyll, fat composition and amino acid composition. Statistical comparison with STAT-ITCF software was made on the values of two replicate analyses, except in the case of xanthophyll, fatty acids and amino acids, where data points are the result of a single analysis.

(i) Compositional analyses

All values for chemical composition were within the normal range for data obtained by the Association Générale des Producteurs de Mais (AGPM), except for total nitrogen, which was higher than normal for both the control and genetically modified corn plants (Table 6).

Protein levels were higher than the normal range for all plants assessed. As protein content is affected by soil nitrogen, it is possible that the fertiliser used in culturing the plants caused the high level of nitrogen for all plants in the study.

Table 6: Summary of compositional analysis for Bt-11 and control corn plants[1].

	Inbred line H8540-Bt	***Isogenic control H8540***	**Hybrid Bt+/Bt-**	***Control hybrid***	**Normal range[2]**
Total nitrogen[3]	13.18 ± 0.07	12.35 ± 0.06	12.28 ± 0.03	12.30 ± 0.07	7.7–10[4]
Moisture	12.3	12.6	12.6	13.3	7–23
Ash	1.47 ± 0.04	1.79 ± 0.007	1.70 ± 0.02	1.6 ± 0.02	1.1–3.9
Starch	68.02 ± 0.4	67.57 ± 0.4	70.83 ± 0.81	70.25 ± 0.48	61–78
Cellulose	2.99 ± 0.007	2.9 ± 0.05	2.67 ± 0.28	2.92 ± 0.05	3.3–4.3 1.93–2.5[4]
Xanthophyll	24.2	21.0	21.6	19.1	19.2–33.1[4]

[1]Samples are 500g of kernels from: Bt^+/Bt^+ H8540 ears n=54, Control H8540 n=56, Bt^+/Bt^- hybrid n=50, Control hybrid ears n= 45. Each data point is the mean of two replicate analyses made with the 500g sample. Data from AGPM. All data except moisture (% H_2O) and xanthophyll (mg/kg dry weight basis) are presented on a % dry weight basis.

[2]Wright, 1987 in Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minnesota, USA.

[3]All values from control and genetically modified lines are significantly different to range.

[4]Data from AGPM

(ii) Amino acid analysis

A summary of amino acid values for plants homozygous for the *cry*1A(b) gene is shown in Table 7. A single analysis was done on 500g samples of kernels from Bt^+/Bt^+ H8540 (number of ears n=54), isogenic control H8540 (number of ears n=56), Bt^+/Bt^- hybrid (number of ears n=50), control hybrid (number of ears n= 45). Values for amino acid composition (once corrected for the high total nitrogen) had minor variations to control values but were within the normal range according to APGM and the published literature. There were no differences in these values greater than 10% (which allows for experimental error) between the modified corn and isogenic controls. The levels of glutamine, asparagine, tryptophan were not determined. No spectrum or literature ranges were available for some of the amino acids, as some of these analyses are not routinely carried out by the laboratory assaying these samples.

Table 7: Summary of amino acid composition data for Bt-11 corn plants[1].

Amino acid composition	**Bt^+/Bt^+ line H8540**	***Control H8540***	**Bt^+/Bt^- hybrid line**	***Control hybrid line***	**Range[2]**
Aspartic acid	9.8	9	8.7	8.4	
Threonine	5.2	5	4.9	5	3.2–3.4
Serine	6.6	6.4	6.1	6.1	
Glutamic acid	28.1	25.7	26.2	25.1	
Proline	12	12.5	12	11.4	
Glycine	4.1	4.2	4	4	
Alanine	11.5	10.8	10.8	10.1	
Cysteine	2.4	2.4	2.5	2.7	
Valine	6.5	6.1	5.9	6.2	4.2–4.6
Methionine	2.5	2.5	2.7	2.9	1.8–1.9
Isoleucine	5.2	4.8	4.6	4.6	3.4–3.7
Leucine	19.4	17.5	17.7	17.3	10–11.3
Tyrosine	5.4	4.9	5	4.7	
Phenylalanine	7.2	6.5	6.4	6.3	4.4–4.5
Lysine	3.2	3.3	3.1	3	2.45–2.6
Histidine	3.5	3.4	3.4	3.5	
Arginine	4.4	4.8	4.9	4.8	4.1–5.2

[1]Values are expressed as g/Kg dry matter.

[2]Data from L'alimentation des animaux monogastriques: porc, lapin, volailles. INRA 1989, Feedstuffs ingredient analysis table, edition 1996, AEC Table and 1995 UCAAB data.

(iii) Fatty acid analysis

A summary of fatty acid values for plants homozygous for the *cry*1A(b) gene is shown in Table 8. A single analysis was done on 500g samples of kernels from Bt^+/Bt^+ H8540 (number of ears n=54), isogenic control H8540 (number of ears n=56), Bt^+/Bt^- hybrid (number of ears n=50), control hybrid (number of ears n= 45). Values for fatty acid composition had minor variations to control values but were within the normal range according to APGM and the published literature. There were no differences in these values greater than 10% (which allows for experimental error) between the modified corn and controls. Literature ranges were available for most of the common fatty acids and not the minor ones as analyses of these fatty acids are not routinely carried out.

Table 8: Summary of fatty acid composition data for Bt-11 corn plants[1].

Fatty acid composition	**Bt^+/Bt^+ line H8540**	***Control H8540***	**Bt^+/Bt^- hybrid line**	**C*ontrol* hybrid line**	**Range**
C16 palmitic acid	15.1	14.5	15.3	14.6	6–22[2]
C18 stearic acid	1.7	1.6	1.6	1.5	1–15[2]
C18:1 oleic acid	20.6	21.9	21.8	21.8	14–64[2]
C18:2 linoleic acid	58.9	58.2	58.1	60	19–71[2]; 56–65[3]
C18:3 linolenic acid	1.7	1.7	1.2	1.1	0.5–2[2]
C20 arachidic acid	0.5	0.4	0.4	0.4	
C20:1 gadoleic acid	0.2	0.2	0.2	0.2	
C22: behenic acid	0.2	0.2	0.1	0.1	

[1]Samples are 500g of kernels from: Bt^+/Bt^+ H8540 ears n=54, Control H8540 n=56, Bt^+/Bt^- hybrid n=50, Control hybrid ears n= 45. Values are expressed as % of the analysed fatty acid relative to the total amount of fatty acids.
[2]From Weber, "Lipids of the kernel", Chapter 10 in Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minnesota, USA. data
[3]AGPM

Study 2: Analysis of Bt-11 dent corn grown in USA

Data set 1

An analysis of the major components and nutritional qualities of elite Bt-11 dent corn lines has also been assessed. These lines are derived from the original transformant. Two genetically modified Bt-11 hybrid corn lines and their near-isogenic controls were grown in three field locations in the USA in 1995. Kernels were analysed for percentage of starch, protein, oil and fibre. These components were estimated by near infrared reflectance (NIR) spectroscopy by the Illinois Crop Improvement Association Inc. NIR analyses are methods used by the American Association of Cereal Chemists.

The kernels from insect-protected corn hybrids were comparable to control hybrids for percentage starch, protein, oil and fibre (Table 9) and fell within the normal ranges expected for these components.

Table 9: Summary of compositional analysis for Bt-11 and control corn plants[1].

	X6534CBR	Isogenic control X6514	X7634CBR	Isogenic control X7514	Normal range[2]
Protein	9.89 (9.40-10.60)	9.96 (9.10-11.40)	10.55 (10.24-11.00)	9.68 (8.90-10.94)	6-12
Oil	4.09 (4.00-4.16)	4.11 (4.10-4.13)	4.02 (4.00-4.02)	4.07 (3.80-4.31)	3.1-5.7
Starch	70.09 (68.80-71.07)	70.19 (67.80-71.50)	69.32 (68.60-70.36)	70.36 (69.07-71.40)	61-78
Fibre	2.95 (2.86-3.00)	2.97 (2.92-3.00)	2.93 (2.89-3.0)	2.91 (2.90-2.92))	2.5[3]

[1]Values presented as % dry weight. Values are means of 3 samples taken from 3 locations (i.e. 1 sample/location), ranges are given in brackets. Genetically modified corn lines are denoted CBR and are isogenic to their controls except for the presence of the novel genes.
[2]From Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minnesota, USA.
[3]Average value

Data set 2

A second nutritional study on Bt-11 dent corn that included additional hybrids was done. Three to five ears were picked from the centre two rows of a four row strip plot for each hybrid per two sites within three geographical regions to give a total of six locations per hybrid. Two of the hybrids are 'northern' (early-season) hybrids and two were 'southern' (mid-late-season) hybrids and were grown with their respective isogenic controls. The grain was analysed by the Illinois Crop Improvement Association using Near Infrared Reflectance Spectroscopy (NIRS) according to methods of the American Association of Cereal Chemists.

(i) Compositional analyses

The compositional data for the Bt-11 corn (denoted as corn borer resistant – CBR) and control corn plants were analysed for significant differences by Analysis of Variance (SAS GLM procedure). The components measured were % protein, oil, starch and fibre (Table 10). Kernels from the early season (northern hybrids) genetically modified corn hybrids (X4334CBR and X4734CBR) have a significantly lower protein content than kernels from the control corn lines (P=5 and P=1 respectively). All other components were comparable between the Bt-11 corn hybrids and their respective control corn lines.

Table 10: Summary of compositional analysis for Bt-11 and control corn plants from a second field trial[1].

Northern / Early	**X4334CBR**	**Control N4242**	**X4734CBR**	**Control N4640**	**Normal range[2]**
Protein	8.65[3] (8.03-9.11)	9.25 (8.63-9.63)	8.19[4] (7.74-9.16)	8.96 (8.28-9.53)	6-12
Oil	3.17 (2.81-3.73)	3.23 (3.04-3.50)	3.34 (3.36-3.48)	3.30 (3.12-3.68)	3.1-5.7
Starch	72.93 (71.8-73.2)	72.57 (71.7-73.4)	72.73 (71.5-73.7)	72.62 (71.3-73.2)	61-78
Fibre	2.69 (2.66-2.83)	2.75 (2.67-2.93)	2.77 (2.68-2.83)	2.77 (2.69-2.83)	2.5[5]
Southern / Mid-late	**X6534CBR**	**X6514**	**X7634CBR**	**X7514**	
Protein	9.52 (8.35-10.60)	9.93 (9.10-11.40)	9.85 (8.63-11.00)	9.87 (8.67-10.94)	6-12
Oil	3.80	3.93	3.37	3.48	3.1-5.7

	(3.63-4.16)	(3.27-4.13)	(2.59-4.00)	(2.70-4.31)	
Starch	70.77 (68.8-72.5)	71.07 (67.8-72.7)	71.33 (68.6-74.3)	71.12 (69.1-73.9)	61-78
Fibre	2.78 (2.55-3.00)	2.80 (2.61-3.0)	2.74 (2.53-3.00)	2.72 (2.46-2.92)	2.5[5]

[1]Values presented as % dry weight. Values are means of a total of 6 samples taken from 2 sites in 3 locations (i.e. 2 distinct samples from each of the 3 locations), ranges are given in brackets.
[2]From Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minnesota, USA.
[3]Values are significantly different to that of control value at 5% level of probability.
[4]Values are significantly different to that of control value at 1% level of probability.
[5]Average value

The "northern" and "southern" hybrids were derived from separate backcross conversion processes using the same original transformation event (plant). Although the protein was lower in the northern hybrids, there is a lack of consistent differences between the non-modified hybrids and their genetically modified equivalents. This may indicate that the effects observed, are not likely to be a result of the genetic modification itself but more likely from differences arising out of an incomplete backcross conversion in the normal breeding process. Values for all the parameters measured fell within the ranges cited in the literature (refer to Table 10).

(ii) Amino acid analyses

Amino acid analyses were performed on kernels obtained from two Bt-11 hybrid corn lines: X6534CBR (a mid-late maturity variety) and X4734CBR (an early maturity variety) and their genetically equivalent controls (N6800 and N4640 respectively). The kernels were sampled from two locations, three samples per line. For further comparison, kernels from another seven non-modified reference hybrids were grown in one of the field trial locations (N4242, N5220, N5866, N6223, N6822, N7070 and N790). Two separate statistical analyses were performed — the first to analyse the variation between hybrids to determine whether there were significant differences between hybrids. The second study analysed differences specifically between genetically modified hybrids and their near-isogenic controls (Table 11).

The first statistical analysis determined the variation between hybrids. Since not all hybrids were replicated, the analysis used the variation observed in hybrids with multiple replicates as an indication of "error" for the other hybrids.
The rationale for this is that other hybrids would have been equally variable. There were significant differences between the hybrids for all values except that for tyrosine (P=5).

Small but significant differences at the 5% level were found between the genetically modified corn hybrid X4734CBR and its control line N4640 for arginine and cysteine. This difference is not consistent for all genetically modified corn hybrids and is consistent with the variability that is observed between lines. Some variability may arise as a result of incomplete backcrossing. This variation is not considered to be a result of the genetic modification nor is it biologically significant.

Table 11: Amino acid profile for Bt-11 hybrids and control corn plants[1].

	X6534-CBR	N6800	X4734-CBR	N4640	N4242[4]	N5220[5]
Tryptophan	0.05-0.06	0.05-0.06	0.05[2]	0.05-0.06	0.05[2]	0.07
Aspartic Acid	0.61-0.67	0.60-0.66	0.54[2]	0.55-0.57	0.55[2]	0.64
Threonine	0.35-0.38	0.35-0.38	0.29-0.30	0.30-0.31	0.30-0.32	0.36
Serine	0.50-0.55	0.50-0.55	0.42-0.43	0.43-0.44	0.43-0.44	0.52
Glutamic Acid	1.54-1.72	1.55-1.79	1.17-1.25	1.22-1.30	1.30-1.32	1.63
Proline	0.77-0.88	0.83-0.91	0.68-0.70	0.63-0.68	0.61-0.66	0.84
Glycine	0.34-0.37	0.35[2]	0.29-0.30	0.31-0.33	0.32-0.34	0.36
Alanine	0.75-0.82	0.75-0.87	0.60-0.62	0.58-0.63	0.61-0.63	0.74
Cysteine[3]	0.21-0.22	0.22-0.23	0.17[2]	0.20-0.21	0.18-0.21	0.22
Valine	0.41-0.43	0.40-0.45	0.32-0.33	0.32-0.34	0.32-0.36	0.43
Methionine	0.19-0.21	0.19-0.22	0.17-0.20	0.20-0.23	0.19-0.21	0.24
Isoleucine	0.28-0.32	0.28-0.33	0.23-0.25	0.24-0.26	0.23-0.27	0.32
Leucine	1.23-1.37	1.23-1.45	0.93-0.98	0.96-0.98	0.92-1.01	1.32
Tyrosine	0.14-0.18	0.14-0.16	0.13[2]	0.13-0.14	0.14[2]	0.17
Phenylalanine	0.44-0.49	0.44-0.51	0.37-0.39	0.36-0.40	0.35-0.38	0.50
Histidine	0.32-0.35	0.34-0.37	0.26-0.27	0.28-0.29	0.27-0.28	0.31
Lysine	0.25-0.26	0.24-0.26	0.23-0.24	0.24-0.25	0.23-0.25	0.27
Arginine[3]	0.36-0.37	0.37-0.38	0.31-0.32	0.32-0.34	0.33[2]	0.39

	N5866[5]	N6223[5]	N6822[5]	N7070[5]	N7590[5]	Range[6]
Tryptophan	0.06	0.07	0.06	0.06	0.08	
Aspartic Acid	0.58	0.68	0.59	0.71	0.67	
Threonine	0.34	0.38	0.34	0.40	0.39	0.32-0.34
Serine	0.47	0.55	0.45	0.53	0.56	
Glutamic Acid	1.54	1.83	1.53	1.61	1.83	
Proline	0.77	0.93	0.79	0.75	1.03	
Glycine	0.34	0.36	0.33	0.40	0.36	
Alanine	0.73	0.85	0.70	0.90	0.83	
Cysteine	0.22	0.22	0.20	0.21	0.23	
Valine	0.40	0.45	0.39	0.48	0.47	0.42-0.46
Methionine	0.23	0.26	0.24	0.27	0.34	0.18-0.19
Isoleucine	0.31	0.35	0.30	0.33	0.34	0.34-0.37
Leucine	1.24	1.46	1.20	1.28	1.47	0.10-0.11
Tyrosine	0.15	0.17	0.16	0.15	0.16	
Phenylalanine	0.47	0.54	0.46	0.46	0.54	0.44-0.45
Histidine	0.31	0.35	0.30	0.37	0.32	
Lysine	0.26	0.26	0.25	0.32	0.25	0.25-0.26
Arginine	0.36	0.40	0.36	0.36	0.38	0.41-0.52

[1]Values are ranges for three samples taken from 3 field sites (i.e. 1 sample/site) and are expressed as g/100g dry weight.
[2]The same value was obtained for all three samples.
[3]Values for genetically modified corn plants are significantly different to those of control corn plants.
[4]Range is obtained from two values
[5]Single value only.
[6]Data from L'alimentation des animaux monogastriques: porc, lapin, volailles. INRA 1989, Feedstuffs ingredient analysis table, edition 1996, AEC Table and 1995 UCAAB data.

(iii) Analysis of fatty acid profiles

Fatty acid analyses were also done on the kernels sampled as described above. The kernels were sampled from two locations, three samples per line from two Bt-11 hybrid corn lines X6534CBR and X4734CBR and their genetically equivalent controls (N6800 and N4640 respectively). Additionally, grain from another seven non-modified reference hybrids were also analysed. As outlined above for the amino acid analysis, two separate statistical analyses

were performed — the first to analyse the variation between hybrids to determine whether there were significant differences between hybrids. The second study analysed differences specifically between genetically modified hybrids and their isogenic controls. The results are shown in Table 12.

A statistical analysis to determine the variation between hybrids, as described above for the amino acid analysis, found no significant differences between the hybrids for fatty acid values (P=5).

Table 12: Fatty acid profile for Bt-11 hybrids and control corn plants[1].

	Palmitic	**Stearic**	**Oleic**	**Linoleic**	**Linolenic**
X6534CBR	10.99-11.14	1.99-2.16	27.15-27.36	56.88-57.31	1.16-1.25
N6800	10.78-11.11	2.11-2.24	26.85-26.90	56.81-57.07	1.29-1.43
X4734CBR	10.76-10.97	2.38-2.41	25.93-26.04	57.62-57.86	1.61-1.67
N4640	10.61-10.65	2.45-2.52	26.31-27.06	56.69-57.59	1.56-1.59
N4242[2]	10.76-11.27	2.15-2.31	25.51-25.89	57.32-57.85	1.59-1.66
N5220[3]	13.14	1.89	26.55	55.13	1.40
N5866[3]	9.17	2.18	21.05	64.53	1.28
N6223[3]	11.53	2.01	26.58	57.04	1.24
N6822[3]	12.05	2.27	18.79	64.30	1.18
N7070[3]	10.11	1.77	25.49	59.77	1.19
N7590[3]	9.86	2.17	20.59	64.68	1.18
Range[4]	6–22	1–15	14–64	19–71 56–65[5]	0.5–2

[1]Values are ranges for three samples taken from 3 field sites (i.e. 1 sample/site) unless otherwise indicated and are expressed as % of fatty acid as a proportion of total fatty acid.
[2]Values are the range for two samples.
[3]Single values given only.
[4]From Weber, "Lipids of the kernel", Chapter 10 in Corn chemistry and technology, 1987, Watson SA and Ramstad PE (eds), American Association of Cereal Chemists, St. Paul, Minnesota, USA. data
[5]Data from AGPM.

A second statistical analysis of the fatty acid values investigated specifically differences between the genetically modified corn hybrid plants versus the non-modified control hybrids. Small but significant differences at the 5% level were observed for palmitic acid (higher in the genetically modified corn line) and stearic acid (lower in the genetically modified corn line). Using the information from the first analysis on the variation that exists between hybrids, the values determined for the Bt-11 hybrids fall within the range determined for the control hybrids. Additionally, all values are within the range reported in the literature (see Table 7).

(iv) Vitamins and minerals

One-pound (2.24 kg) samples of grain were taken from each of three locations from two Bt-11 corn hybrids N4242-Bt and N4640-Bt and their corresponding near-isogenic non-modified hybrids and analysed for their vitamin and mineral content. The grain was analysed for the minerals copper, magnesium, manganese and zinc as well as the vitamins folic acid, niacin, vitamin B_1 and vitamin B_2 (Table 13). No significant differences (p=0.05) between Bt-11 corn hybrids and their corresponding control hybrids were observed for any of the selected components.

Table 13: Vitamin and mineral profile for Bt-11 and control corn plants[1].

	N4242Bt	Control N4242	N4640Bt	Control N4640
Copper	0.17 ± 0.06	0.17 ± 0.06	0.20 ± 0.0	0.20 ± 0.0
Magnesium	95.7 ± 1.15	91.7 ± 5.51	90.0 ± 1.73	86.3 ± 4.73
Manganese	0.47 ± 0.06	0.43 ± 0.06	0.40 ± 0.0	0.33 ± 0.06
Zinc	1.93 ± 0.06	2.03 ± 0.29	1.77 ± 0.12	1.70 ± 0.10
Folic acid	0.051 ± 0.010	0.045 ± 0.002	0.57 ± 0.03	0.57 ± 0.03
Niacin	8.62 ± 1.32	8.03 ± 0.14	8.96 ± 0.21	9.49 ± 0.41
B_1	1.44 ± 0.10	1.37 ± 0.21	1.26 ± 0.23	1.48 ± 0.15
B_2	0.71 ± 0.04	0.70 ± 0.09	0.72 ± 0.04	0.71 ± 0.02

[1]Values are means of 3 samples, one from each of 3 locations. Minerals are expressed as % and vitamins are expressed in mg/lb.

Study 3: Comparison of nutritional composition of fresh and canned Bt-11 sweet corn

A fourth analysis of Bt-11 corn lines was done, specifically to assess the nutritional value of three Bt-11 sweet corn varieties. Corn was harvested from the Bt-11 sweet corn hybrids, Bt 98-0943, Bt 95-0937 and Bt 95-0941, and from their corresponding near-isogenic non-modified hybrids, grown in 1996 at one location in the United States. Ten ears of each of the hybrids were harvested at prime harvest and analysed as fresh corn on the cob. Corn from each hybrid was canned and also analysed (processed corn analysis).

Fresh and canned sweet corn was analysed for moisture, protein, fat, ash, carbohydrates, fibre, vitamins and minerals (Table 14) according to methods from the Association of Official Analytical Chemists. Given that there was only duplicate analysis of the one sample taken for each line, no statistical analysis was performed.

Comparable nutritional composition was observed between the three Bt-11 sweet corn hybrids and their corresponding isogenic hybrids for both the fresh corn and canned corn.

Table 14: Compositional profile for fresh and canned sweet corn Bt-11 hybrids[1].

Fresh	**Bt 95-0943**	**Jubilee**	**Bt 95-0937**	**Bonus**	**Bt 95-0941**	**Empire**
Moisture (g)	69.88 – 69.78	69.67-69.70	73.65	72.20-72.24	71.15-71.28	70.34-70.56
Protein (g)	3.7-4.09	3.20-4.35	3.75-3.37	3.89-4.06	3.75-3.83	4.17-4.26
Fat (g)	0.76-1.34	1.10-0.97	0.75-0.91	0.81-0.88	0.85-1.18	0.91-1.13
Ash (g)	0.90-0.93	0.91	0.99-1.05	1.00-1.03	1.01-1.02	0.91-0.95
Carbohydrates - total[2] (g)	24.28	24.63	20.94	22.06	22.89	23.36
Calories[2]	111	112	93	100	105	110
Calories[2] from fat	10	9	7	7	10	9
Sugars[2] (g)	6.8	6.31	4.14	4.38	5.21	4.86
Other Carbohydrates[2] (g)	14.71	15.59	13.77	15.01	14.81	16.04
Total Dietary Fibre (g)	2.83-2.71	2.93-2.54	2.61-3.44	2.64-2.70	2.36-3.38	2.38-2.54
Vitamin A[2] (IU)	230	137	280	211	95.8	160
Vitamin C[2] (mg)	0.869	1.63	7.35	6.53	7.25	7.69
Sodium (mg)	9.9-14.2	5.9-7.2	10.0-13.0	3.9-5.3	5.8-7.2	4.9-8.6
Potassium (mg)	293.5-286.2	326.0-322.6	287.6-307.4	292.6-306.7	372.7-391.8	255.6-322.9
Calcium (mg)	3.4-8.6	1.6	0.7-7.1	0.0-0.4	7.1-8.0	0.7-7.1
Iron (mg)	0.49-0.85	0.49-0.56	0.57-0.61	0.6-0.90	0.54-0.63	0.71-0.74
Canned	**Bt 95-0943**	**Jubilee**	**Bt 95-0937**	**Bonus**	**Bt 95-0941**	**Empire**
Moisture (g)	77.81 – 77.83	76.81-76.85	77.66-77.76	77.77-77.80	76.44-76.52	77.80-77.96
Protein (g)	2.95-2.99	2.62-2.97	2.95-3.00	3.09-3.18	2.85-2.94	2.93-3.02
Fat (g)	0.85-1.77	1.02-1.90	1.01-1.09	0.68-0.75	0.83-0.96	0.62-0.85
Ash (g)	0.97-1.01	1.01	0.84-0.85	0.85-0.87	0.85-0.87	0.83-0.83
Carbohydrates - total[2] (g)	16.91	17.92	17.42	17.5	18.87	17.59
Calories[2]	83	87	81	79	86	79
Calories[2] from fat	12	13	9	6	8	7
Sugars[2] (g)	1.8	1.92	1.54	1.3	1.89	1.53
Other Carbohydrates[2] (g)	12.99	13.85	13.38	13.72	14.65	13.56
Total Dietary Fibre (g)	1.99-2.23	2.01-2.29	2.47-2.55	2.41-2.54	2.19-2.48	2.18-2.82
Vitamin A[2] (IU)	175	209	192	185	175	206
Vitamin C[2] (mg)	2.07	2.32	2.25	2.31	2.15	1.99
Sodium (mg)	262.8-285.0	266.1-304.1	245.9-248.0	212.5-230.2	225.7-239.6	191.9-235.6
Potassium (mg)	199.9-202.8	212.2-262.4	210.3-228.4	191.4-202.6	181.1-205.3	176.3-200.2
Calcium (mg)	3.1-8.8	2.4-4.2	0.0-1.8	5.1-8.2	3.7-10.2	5.2-8.2
Iron (mg)	0.29-0.55	0.289-0.614	0.31-0.25	0.23-0.34	0.348-0.387	0.31-0.37

[1]Values are expressed per 100 g serving basis.

[2]Only one sample determine

Study 4: Analysis of Bt-11 dent corn lines treated with herbicide

An additional study was done to assess the potential effects of herbicide treatment on the major components of the corn kernels. Three Bt-11 hybrids representing different maturity types (Madera, Manuel and Magister) and their isogenic controls were grown in open fields at two locations in France in 1998. Proximate analysis (carbohydrate, protein, fat and fibre), fatty acids and amino acid composition were compared between transgenic crops treated with a glufosinate ammonium herbicide (Liberty®) at a rate of 2.25 L/ha active ingredient at the 3 and 6–7 leaf stages and untreated transgenic and isogenic controls (Table 15). Values presented in this experiment are not directly comparable to values for other experiments because they have been performed by a different laboratory using slightly different methods.

(i) Compositional analyses

No significant differences in composition were found between the treated Bt-11 corn plants and untreated Bt-11 corn plants nor between the untreated Bt-11 corn plants and the unmodified control corn plants (P=5).

Table 15: Compositional analyses for Bt-11 hybrids and control corn plants[1].

	Treated	Untreated	Control
Energy	1441 ± 37	1430 ± 35	1433 ± 29
Carbohydrate	70.0 ± 2.0	69.5 ± 1.5	68.8 ± 1.5
Protein	7.6 ± 0.9	8.2 ± 0.8	8.4 ± 0.8
Fat	3.3 ± 0.6	3.0 ± 0.6	3.3 ± 0.8
Fibre	8.0 ± 1.0	8.0 ± 0.8	7.7 ± 0.2

[1]Values are means of 3 samples, one from each of the hybrids Madera, Manuel and Magister. Values are all expressed as a % except for energy (KJ/100g).

(ii) Amino acid analysis

Amino acid levels were also analysed (Table 16a). The values for cysteine and tryptophan were not determined. Using the F test, significantly different values were obtained for glutamic acid, proline, alanine, isoleucine and phenylalanine when comparing all three treatments (treated GM, untreated GM and control) (at the P=5 level). In a comparison of the values for treated Bt11 hybrids to the non-modified control hybrids, only the values for proline and alanine were significantly different (lower in treated Bt-11 hybrids than in the control lines).

A breakdown of the values for proline and alanine for each of the three hybrids is shown in Table 16b. The difference between the treated modified and non-modified line was not consistent for all lines and may be a result of variability between the lines. This difference is not considered to raise safety or nutritional concerns.

Table 16a: Amino acid analyses for Bt-11 hybrids and control corn plants[1].

	Treated	Untreated	Control
Aspartic Acid	4690 ± 406	5033 ± 439	4703 ± 142
Threonine	2690 ± 423	2850 ± 165	2690 ± 423
Serine	3537 ± 353	3750 ± 260	3537 ± 353
Glutamic Acid	14533 ± 1595	16233 ± 1626	15700 ± 625
Proline	6967 ± 1154	8367 ± 234	8590 ± 769
Glycine	3047 ± 238	3187 ± 111	2920 ± 26
Alanine	5057 ± 415	5760 ± 606	5500 ± 207
Valine	2963 ± 552	3327 ± 654	3210 ± 183
Methionine	1030 ± 183	1270 ± 122	1170 ± 30
Isoleucine	1717 ± 315	2320 ± 368	2013 ± 42
Leucine	8153 ± 918	9320 ± 1105	8787 ± 420
Tyrosine	3800 ± 573	4240 ± 455	3957 ± 172
Phenylalanine	3163 ± 440	3540 ± 243	3363 ± 280
Histidine	1867 ± 376	2147 ± 170	1853 ± 169
Lysine	1967 ± 228	2223 ± 228	1967 ± 163
Arginine	3257 ± 319	3443 ± 119	3160 ± 236

[1]Values are means of 3 samples, one from a different maturity type. Values expressed as mg/kg.
[2]Data from L'alimentation des animaux monogastriques: porc, lapin, volailles. INRA 1989, Feedstuffs ingredient analysis table, edition 1996, AEC Table and 1995 UCAAB data.

Table 16b: Significant differences in amino acid profiles between treated genetically modified hybrids and non-genetically modified hybrids[1].

Hybrid	Proline (mg/kg)		Alanine (mg/kg)	
	Bt11 hybrid[2]	Control hybrid	Bt11 hybrid[2]	Control hybrid
Madera	5640	7730	4720	5330
Manuel	7520	9210	5520	5730
Magister	7740	8830	4930	5440

[1]Values are all expressed as mg/kg.

(iii) Fatty acid analysis

Fatty acid levels were also analysed. No significant differences were found between fatty acid values for treated and untreated genetically modified corn plants and also between the untreated modified plant and control lines (P=5%) (Table 17).

Table 17: Fatty acid analyses for treated Bt-11 plants and control corn plants[1].

	Treated	Untreated	Control
Palmitic	12.4 ± 1.9	12.3 ± 1.2	11.2 ± 1.2
Stearic	2.3 ± 0.2	2.4 ± 0.3	2.2 ± 0.2
Oleic	28.0 ± 1.9	27.4 ± 2.0	27.2 ± 1.3
Linoleic	55.1 ± 2.7	55.8 ± 3.0	57.0 ± 2.3
Linolenic	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2

[1]Values expressed as a % of total fatty acids. Values are means of 3 samples, one from each of the hybrids Madera, Manuel and Magister.

Levels of anti-nutrients

Corn contains few natural toxins or anti-nutrients. The anti-nutrients trypsin and chymotrypsin inhibitors are present in corn at very low levels and are not considered nutritionally significant (Wright 1987).

Ability to support typical growth and well-being

In assessing the safety of a genetically modified food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further reassurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

The compositional and other data presented in the application are considered adequate for establishing the nutritional adequacy of Bt-11 corn. Additional studies, including animal feeding studies are therefore not required.

Other information – dietary exposure assessment

Dietary exposure to the Cry1Ab protein from consumption of Bt-11 corn has been estimated. The exposure to the PAT protein was not determined because it is essentially negligible in corn kernels (i.e. at the limit of detection).

The *Bt* protein is not considered toxic to mammals, including humans. Because of the absence of any hazard, an estimate of the dietary intake of the *Bt* protein was not considered essential for the safety assessment. However, it is recognised that such information may be useful in providing reassurance to the community that exposure to a novel protein is low and/or that the novel protein is likely to be present in the diet at levels well below those found to be safe in animal toxicity studies.

Cry1Ab is expressed in Bt-11 corn kernels at levels ranging from 0.78 to 3.80 µg protein/g fresh weight. Therefore, if certain assumptions are made about market penetration of the Bt-11 corn products, it is possible to estimate the dietary intake of the *Bt* protein.

Australian and New Zealand consumption data is available for maize flour and products in which maize flour is an ingredient (corn flour, corn meal: raw, cooked with water and cooked with milk, custard powder, breakfast flakes, breakfast puffed, tortilla, taco shells, pasta). Although other corn products exist, the above corn products represent the major processed corn products available on the market and are also more likely to be present in the corn based food or food ingredients imported from the USA and Canada (eg corn flour). It should be noted that these estimates assume that all corn products consumed in Australia and New Zealand are made using Bt-11 corn and will therefore be an overestimate of the true content of Bt-11 corn. Data on the dietary intake of other processed corn products is not available (eg high fructose corn syrup).

Excluding other corn products, the average total consumption[2] of processed corn products per person is 3.48 g/day in Australia, and 3.23 g/day in New Zealand. If, however, the consumption figures are based only on those in the population who report consuming such corn products, then the average total consumption is 20.0 g/day and 14.1 g/day in Australia and New Zealand respectively and the 97.5th percentile consumption is 90 g/day and 68 g/day in Australia and New Zealand respectively.

For calculation of the dietary intake of the novel proteins, the highest corn product consumption figure (90 g/day) and the highest Cry1Ab protein concentrations (3.80 µg protein/g fresh weight) was used. This represents a 'worst-case' estimate.

To do the calculation, assumptions about the proportion of processed corn products derived from Bt-11 and Bt-176 corn must be made. In 2000, Bt-11 and Bt-176 comprised less than 6% (4.2 and 1.4% respectively) of the total United States corn acreage (NASS, USDA 2000[3]). It is possible therefore to make two dietary intake estimates: one using a very worst case estimate where it is assumed that all corn products on the market are derived entirely from the two Bt corn lines; and the more realistic but still conservative estimate, where it is assumed that 10% of corn products are derived from Bt-11 corn and Bt-176. The dietary intake estimates are provided in the table below:

Theoretical Market penetration	**Estimated dietary intake of Cry1Ab**	
	µg /day	**µg/kg bw/day[1]**
100 %	342	5.10
10 %	34.2	0.510

[1] assuming a body weight of 67 kg.

The worst-case estimate of dietary exposure is at least 0.7 million times less than the dose found to have no adverse effects in mice (3535 mg Cry1Ab/kg body weight). Therefore, even if all processed corn products were to be derived from Bt-11 and Bt-176 corn, a very large margin of safety exists.

Conclusions regarding nutritional issues

The nutritional qualities of insect-protected Bt-11 corn were determined by compositional analyses of the major and minor components of the kernels and these were found to be comparable in all respects to the conventional corn lines. Additionally, a dietary exposure assessment demonstrates that exposure to the novel protein from Bt-11 corn is likely to be small.

Based on the data submitted in the present application, grain derived from Bt-11 corn is nutritionally and compositionally comparable to that from conventional corn and is not considered to pose a risk to human health and safety.

[2] Calculated for all respondents

[3] Crop Production, 9 November 2000. National Agricultural Statistics Service, US Department of Agriculture.

ACKNOWLEDGEMENTS

ANZFA gratefully acknowledges Dr Brian Jordan, Associate Professor, Director, Nutrition and Health, Institute of Food Nutrition and Human Health, Massey University, New Zealand, for expert comments on this safety assessment report.

REFERENCES

Alexander, R.J. 1987 Corn dry milling: processes, products and applications. In: *Corn: Chemistry and Technology*. ed. Watson, S.A. and Ramstead, P.E. American Association of Cereal Chemists Inc, St Paul, Minnesota. pp 351-376

ANZFA 1999. Guidelines for the safety assessment of foods to be included in Standard A18 — Food Produced Using Gene Technology.

Calva JJ, Sifuentes-Osbornio J and Ceron C 1996. Antimicrobial resistance in fecal flora: longitudinal community-based surveillance of children from urban Mexico. Antimicrobial Agents and Chemotherapy 40:1699-1701.

Frick, O.L. 1995 The potential for allergenicity in transgenic foods. In: *Genetically Modified Foods: Safety Aspects*. K.-H. Engel, G.R. Takeoka and R. Teranishi. (eds) American Chemical Society, Washington DC.

Geiser, M., Scweitzer, S. and Grimm, C. 1986 The hypervariable region in the genes encoding entomopathogenic crystal proteins of *Bacillus thuringiensis*: nucleotide sequence of the kurhd1 gene of subsp. *kustaki* HD1. *Gene* 48: 109-118

Hammond BG and Fuchs RL 1998. Safety evaluation for new varieties of food crops developed through biotechnology. In: Thomas JA (ed.) *Biotechnology and safety assessment*. Taylor and Francis, Philadelphia.

Hofte, H. and Whitely, H.R. 1989 Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiol Rev* 53: 242-255.

INRA. 1989. L'alimentation des animaux monogastriques: porc, lap In: *Volailles. Feedstuffs Ingredient Analysis Table,* edition 1996, AEC Table and 1995 UCAAB data

Jones DD and Maryanski JH 1991. Safety considerations in the evaluation of transgenic plants for human food. In: Levin MA and Strauss HS (eds) *Risk assessment in genetic engineering*. New York: McGraw-Hill.

Kues U and Stahl U 1989. Replication of plasmids in gram-negative bacteria. Microbiological Reviews 53:491-516.

Lehrer, S.B., W.E. Horner and Reese, G. 1996. Why are some proteins allergenic? Implications for biotechnology. *Crit Rev Food Sci Nutr 36*: 553-564.

Lehrer SB and Reese G 1998. Food allergens: implications for biotechnology. In: Thomas JA (ed.) Biotechnology and safety assessment. Taylor and Francis, Philadelphia.

Mascarenhas D, IJ Mettler, DA Pierce and HW Lowe. 1990. Intron-mediated enhancement of heterolgous gene expression in maize. *Plant Mol. Biol*. 15:913-920

May, J.B. 1987 Wet milling: processes and products. In: *Corn: Chemistry and Technology.* ed. Watson and Ramstead. American Association of Cereal Chemists Inc, St Paul, Minnesota. pp 377-397

Negrutiu I, R Shillito, I Potryjus, G Biasini and G Sala. 1987. Hybid genes in the analysis of transformation conditions. Plant Molec. Biol. 8:363-373.

Noteborn HP, Bienenmann-Ploum ME, van den Berg JH, Alink GM, Zolla L, Reynaerts A, Pensa M and Kuiper HA 1995. Safety assessment of the Bacillus thuringiensis insecticidal crystal protein Cry1A(b)expressed in transgenic tomatoes. In: Genetically modified foods. American Chemical Society Symposium Series 605. Engal K-H, Takeoka GR and Teranishi R (eds) American Chemical Society, Washington, DC.

Perlak, FJ, Fuchs, RL, Dean, DA, McPherson, SL, and Fischhoff, DA. 1991 Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc Natl Acad Sci USA* 88: 3324-3328

Rogers, J. 1990 *What food is that and how healthy is it?* Weldon Publishing, Sydney p 326-327

Sanders PR, Lee TC, Groth ME, Astwood JD and Fuchs RL 1998. Safety assessment of insect-protected corn. In: Thomas JA (ed.) *Biotechnology and safety assessment*. Taylor and Francis, Philadelphia.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. 1998 *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62: 775-806

Stewart GJ and Carlson CA 1986. The biology of natural transformation. Annual Review of Microbiology, 40:211-235.

Strauch E., Wohlleben, W. and A. Puhler. 1988. Cloning of a phosphinothricin N-acetyl transferase gene from *Streptomyces viridochromogenes* Tü4994 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene 63:65-77

Taylor S.L. and S.B. Lehrer. 1996. Principles and characteristics of food allergens. *Crit Rev Food Sci Nutr* 36 Suppl: S91-S118.

Thompson, C.K., Rao Movva, N., Tizard, R., Crameri, R., Davies, J.E., Lauwereys, M. and Botterman, J. 1987 Characterization of the herbicide resistance gene *bar* from *Streptomyces hygroscopicus.* *EMBO J* 6: 2519-2623

Weber, E.J. 1987 Lipids of the kernel. In: *Corn: Chemistry and Technology*. ed. Watson, S.A. and Ramstead, P.E. American Association of Cereal Chemists Inc, St Paul, Minnesota. pp 311-349

Wehrmann, A., A. Van Vliet, C. Opsomer, J. Botterman and A. Schulz. 1996. The similarities of bar and pat gene products make them equally applicable for plant engineers. *Nature Biotechnology* 14:1274-1278

WHO 1991 *Strategies for assessing the safety of foods produced by biotechnology*. Report of a joint FAO/WHO Consultation. World Health Organization, Geneva, 59 pp.

WHO 1993 *Health aspects of marker genes in genetically modified plants*. Report of a WHO Workshop. World Health Organization, Geneva, 32 pp.

Wohlleben, W., Arnold, I. Broer, D. Hilleman, E. Strauch and. 1988. Nucleotide sequence of the phosphinothricin-N-acetyltransferase gene from a *Streptomyces viridochromogenes* T494 and its expression in *Nicotiana tabacum*. Gene 70:25-37.

Wright, K.N. 1987 Nutritional properties and feeding value of corn and its by-products. In: *Corn: Chemistry and Technology*. ed. Watson, S.A. and Ramstead, P.E. American Association of Cereal Chemists Inc, St Paul, Minnesota. pp 447-478