FOOD PRODUCED FROM INSECT - PROTECTED Bt-176 CORN

A Safety Assessment

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SUMMARY

Food derived from corn line Bt-176 has been evaluated for its suitability for human consumption. The evaluation criteria included analysis of changes at the DNA, protein and whole food levels, and an 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

Insect-protected Bt-176 corn was generated by the transfer of the *cry1A(b)* gene derived from *Bacillus thuringiensis* subsp *kurstaki* which confers protection against attack by insects. The Cry1A(b) protein is an insecticidal crystal protein, whose toxic effect is specific to Lepidopteran insects, including the European Corn Borer. The introduced gene for *cry1A(b)* was found to be stably integrated into the corn plant genome and is phenotypically and genetically stable over multiple generations.

Other genes transferred with the *cry1A(b)* gene were the *bar* gene and the *bla* gene. The *bar* gene was used as a marker to select transformed plant cells during the corn transformation procedure. It codes for the enzyme phosphinothricin acetyltransferase (PAT) and is derived from the bacterium *Streptomyces hygroscopicus*. It confers resistance to the herbicide phosphinothricin (glufosinate ammonium) and was used as a selectable marker for transformed plants. The level of PAT expressed in Bt-176 corn was at least thirty times less than in phosphinothricin-tolerant corn lines developed by Novartis for agronomic use, and Bt-176 corn is not intended to be marketed as a herbicide-resistant plant. The *bla* gene was used as a marker to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken prior to transformation of the plant cells. It codes for the enzyme β-lactamase and confers resistance to the antibiotic ampicillin.

The molecular and genetic analyses indicated that the introduced genes have been stably integrated into the genome of Bt-176 corn and were stably inherited for 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 a large number and diverse range of foods, and have a long history of safe use. Products derived from Bt-176 corn hybrids may include highly processed corn products such as flour, breakfast cereals, high fructose corn syrup and other starch products as well as fresh sweet corn and associated products.

The transformed corn produces two new proteins: Cry1A(b) and phosphinothricin acetyltransferase (PAT). In kernels, the expression of Cry1A(b) was detected but was below the limit of quantification of 5 ng/g fresh weight and the PAT protein was not detected. The *bla* gene was not expressed in plants.

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 or bacteria in the human digestive tract. Much of the concern in this regard is with the presence of antibiotic resistance genes in genetically modified foods. In the case of the insect-protected Bt-176 corn, it was concluded that the *bla* gene would be extremely unlikely to transfer to bacteria in

the human digestive tract because of the number and complexity of the steps that would need to take place consecutively. More importantly however, in the highly unlikely event that transfer did occur, the human health impacts would be negligible because ampicillin resistant bacteria are already commonly found in the human gut and in the environment. Transfer of novel genetic material from the insect-protected Bt-176 corn to human cells via the digestive tract was also considered to be equally unlikely.

The level of DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one), a naturally occurring plant defence compound, was unaltered in Bt-176 corn indicating that the genetic modification has not altered the levels of these compounds.

Toxicological issues

Corn does not have any naturally-occurring toxins or allergens and has a long history of safe use. Cry proteins from *B. thuringiensis* have a long history of safe use as insecticides. In Bt-176 corn kernels, the Cry1A(b) protein is detectable but below the limit of quantification. The PAT protein is not detectable in Bt-176 kernels. The *bla* gene is not expressed in Bt-176 corn.

Data for the newly expressed Cry1A(b) and PAT proteins in Bt-176 corn have been evaluated for their potential toxicity to humans. Studies showed no signs of toxicity among mice following acute oral doses up to 3535 mg/kg for Cry1A(b) and 2575 mg/kg for PAT. No significant similarity to the amino acid sequences of known toxins was identified for either protein.

Neither of the expressed proteins exhibits characteristics of known allergens and amino acid sequence analyses did not reveal any similarities to known allergens. Both proteins have been shown to be rapidly digested in simulated mammalian digestive systems. Therefore, the evidence does not indicate that there is any potential for either protein to be toxic or allergenic to humans.

Nutritional issues

The compositional analyses were comprehensive and demonstrated that there are no substantial differences in the levels of major constituents or nutrients, between Bt-176 corn and conventional corn lines. The components measured were proximate (protein, fat, moisture, fibre, ash, carbohydrates and calories), fatty acids and, amino acids. Additionally, the nutritional adequacy of Bt-176 corn was found to be equivalent to that of conventional corn in a feeding study with chickens.

These analyses confirm that insect protected Bt-176 corn is nutritionally and compositionally comparable to other corn lines and that no health or safety risks are posed by consuming food derived from the genetically modified corn.

Conclusion

No public health and safety concerns have been identified in the assessment of insect-protected Bt-176 corn. Based on the currently available data, food derived from the genetically modified Bt-176 corn is comparable to food derived from conventional corn in terms of its safety and nutritional adequacy.

FOOD PRODUCED FROM INSECT-PROTECTED Bt-176 CORN

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on corn that has been genetically modified to provide protection against certain insects, specifically the European corn borer (ECB). The corn referred to as '*Bt-176* corn' has been modified to confer protection against insect attack by the production of an insecticidal protein representing the active portion of the Cry1A(b) protein that occurs naturally in *Bacillus thuringiensis* subsp. *kurstaki* strain HD1. The insect-protected corn plants are protected against attack from Lepidopteran insects, including the European Corn Borer. The *bar* gene, which confers increased tolerance to the herbicide phosphinothricin, has also been introduced to Bt-176 corn, but it does not confer protection to commercial applications of the herbicide.

The Bt-176 corn described in this application includes insect-protected (dent) corn hybrids into which the Bt-176 transformation event has been introduced. According to the applicant, grain harvested from Bt-176 corn will enter the food chain only after processing. It should be noted that two other varieties of corn are cultivated: sweet corn and popping corn. Sweet corn is the variety grown for use as a fresh vegetable or in canned form. Popping corn is grown for use in the production of popcorn. While the current application refers to the insect-protection trait in dent corn hybrids the germplasm from the Bt-176 event may subsequently be introduced into commercial sweet and popping corn varieties.

Following assessment by the United States Department of Agriculture (USDA) in 1995 hybrids incorporating the Bt-176 event were commercialised in the USA (USDA 1995). The US Food and Drug Administration (US FDA) approved the use of Bt-176 corn in human food in 1995 (US FDA 1999). In 1997 the United States Environmental Protection Agency (US EPA) exempted phosphinothricin acetyl transferase (PAT, and the genetic material, i.e. the *bar* gene, necessary for its production in all plants) from "the requirement of a tolerance on all raw agricultural commodities" (US EPA 1997). Approvals for environmental release and use in human food and animal feed in Canada were given in 1995 and 1996 respectively (Canadian Food Inspection Agency 1995, 1996).

Corn harvested from these plants or processed products containing Bt-176 corn components may have been imported into Australia and New Zealand since 1995. There has been no application for commercial cultivation of Bt-176 corn in Australia or New Zealand.

Domestic production of corn in both countries is supplemented by a small amount of imported corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Other products include maize starch which is used by the food industry for the manufacture of dessert mixes and canned foods and corn-based ingredients processed into breakfast cereals, baking products, extruded confectionary and corn chips.

The data regarding the generation and characterisation of Bt-176 corn and backcross hybrids have been published in peer-reviewed scientific literature (Koziel *et al* 1993, Fearing *et al* 1997, Brake and Vlachos 1998).

DESCRIPTION OF THE MODIFICATION

Methods used in the genetic modification

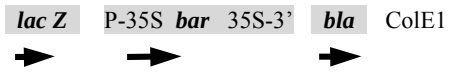
The *Bt-176* corn was produced by simultaneous introduction of plasmids pCIB3064 and pCIB4331 (Figures 1a & b) into immature embryos of proprietary inbred corn line CG00526 (*Zea mays* L.) via microprojectile bombardment.

pCIB3064 contains the *bar* gene for herbicide resistance and the *bla* gene for antibiotic resistance. Schematic maps of the two plasmids are shown in Figures 1a and 1b. pCIB4331 contains two copies of the *cry*1A(b) gene for insect resistance and a single copy of the *bla* gene for antibiotic resistance.

Transformed plants were selected on the basis of their ability to grow in the presence of phosphinothricin conferred by the transfer of the *bar* gene.

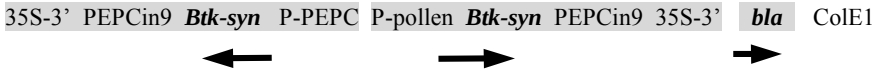
Until recently, transformation of cereals has been achieved by use of the particle bombardment technique rather than the *Agrobacterium*-mediated DNA transformation system (Komari *et al* 1998). Introduction of DNA into the plant is achieved by bombarding tissues with microscopic particles (commonly tungsten or gold) coated with the DNA of interest (Klein *et al* 1992).

Figure 1a Schematic diagram of pCIB3064[1]



[1] See Table 1 for an explanation of the abbreviations. Not to scale.

Figure 1b Schematic diagram of pCIB4431[1]



[1] See Table 2 for an explanation of the abbreviations. Not to scale.

Function and regulation of the introduced genes

A total of four genes were transferred to the corn line. Plasmid pCIB4431 contained two constructs of the *cry1A(b)* gene for expression in corn plants. Plasmid pCIB3064 contained one construct of the *bar* gene for expression in corn plants as well as the *lacZ* gene. The *bla* gene was present on both plasmids. The genetic elements in pCIB3064 and pCIB4431 are shown in Tables 1 and 2 respectively.

The cry1A(b) gene

The *cry1A(b)* gene used to generate Bt-176 corn was derived from the soil bacterium *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*) strain HD1 (Geiser *et al* 1986). The gene encodes a δ-

endotoxin that protects against certain species of Lepidopteran insects including the European corn borer (ECB, *Ostrinia nubilalis*). The *cry*1A(b) gene introduced into Bt-176 corn was truncated version of the native *cry*1A(b) gene equivalent to the coding region for the N-terminal 648 amino acids of the 1155 amino acid full length native Cry1A(b) protein. This 648 amino acid peptide includes the portion responsible for insecticidal activity, and is processed by proteases in the lepidopteran gut to yield an insecticidal protein of 564–578 amino acids.

Until recently δ-endotoxins present in *B. thuringiensis* subspecies were classified into four groups on the basis of their insecticidal range: CryI, Lepidoptera specific; CryII, Lepidoptera and Diptera specific; CryIII, Coleoptera specific; and CryIV, Diptera specific (Hofte and Whitely 1989). Since that time over 100 *cry* genes have been sequenced. Characterisation of many of these *cry* genes is inconsistent or anomalous with the original system and a revised nomenclature was recently proposed (Crickmore *et al* 1998). Under the new nomenclature *cry*1A(b) would be referred to as *cry*1Ab4. However, to retain consistency with the data provided by the applicant, the former nomenclature of *cry*1A(b) has been retained in this assessment.

The native *cry*1A(b) gene contains codons that are not frequently used in plant genes as well as A+T rich regions that could be potential polyadenylation sites thus impairing its expression in the plant. The *cry*1A(b) gene used to transform corn was modified to reflect plant codon usage to allow efficient expression in the corn plant (Perlak *et al* 1991, Koziel *et al* 1993). The synthetic *cry*1A(b) DNA sequence is 65% identical to the native gene, however the encoded amino acid sequence of the resultant Cry1A(b) protein is identical to that of the native toxin (Koziel *et al* 1993, Koziel *et al* 1996).

Plasmid pC1B4431 contains two copies of the synthetic *cry*1A(b) gene; one controlled by the promoter from the corn phosphoenolpyruvate carboxylase (PEPC) gene, specific for expression in the green tissue of the plant (Hudspeth and Grula 1989), the other controlled by a corn calcium-dependent protein kinase gene (P-pollen), resulting in pollen-specific expression (Estruch *et al* 1994). Transcription termination and polyadenylation of mRNA of both copies of *cry1A(b)* are controlled by the 3' untranslated 35S sequence from cauliflower mosaic virus (CaMV). Both *cry1A(b)* gene constructs also contain intron #9 from the corn PEPC gene, which stimulates expression of the gene encoding the truncated Cry1A(b) protein (Callis *et al* 1987).

The bar gene

The *bar* gene is derived from the soil microorganism *Streptomyces hygroscopicus* and confers resistance to the herbicide phosphinothricin.

The *bar* gene was used as a selectable marker to distinguish transformed (i.e. genetically modified) corn cells from unmodified cells. To ensure the expression of the *bar* gene in plant cells it was fused to the 35S promoter and 3' polyadenylation sequences from cauliflower mosaic virus (CaMV, Benfey and Chua 1990) to direct initiation and termination of transcription and polyadenylation of the mRNA transcript.

Transformed callus tissue was selected for on the basis of phosphinothricin tolerance. Putative transformants were further selected by amplification of transferred sequences using the polymerase chain reaction (PCR).

Table 1. Genetic Elements contained in pCIB3064

Genetic element	Region	Name	Function	Source
bla		*bla*	Ampicillin resistance in bacterial cells	*Escherichia coli*
lacZ		*lacZ*	partial coding sequence of lacZ *lacZ* encodes β-galactosidase	*Escherichia coli*
bar	Promoter *bar* 3' untranslated	P-35S *bar* Tr7	drives expression in plant cells Phosphinothricin acetyl transferase signals termination of transcription	Cauliflower Mosaic Virus *Streptomyces hygroscopicus* Cauliflower Mosaic Virus
ColE1		ColE1	origin of plasmid replication in *Escherichia coli*	*Escherichia coli*

Table 2. Genetic Elements contained in pCIB4431

Genetic element	Region	Name	Function	Source
Btk	Promoter Btk-syn Enhancer 3' untranslated	PEP-C *cry1A(b)* PEP-C Intron #9 35S 3'	drives expression in green plant cells Bt toxin enhances transcription signals stop point of transcription and initiation of polyadenylation	*Zea mays* *Bacillus thuringiensis* subsp *kurstaki* *Zea mays* Cauliflower Mosaic Virus
Btk	Promoter Btk-syn Enhancer 3' untranslated	P-Pollen *cry1A(b)* PEP-C Intron #9 35S 3'	drives expression in pollen Bt toxin enhances transcription signals stop point of transcription and initiation of polyadenylation	*Zea mays* *Bacillus thuringiensis* subsp *kurstaki* *Zea mays* Cauliflower Mosaic Virus
bla		*bla*	Ampicillin resistance in bacterial cells	*Escherichia coli*
lacZ		*lacZ*	partial coding sequence of *lacZ* *lacZ* encodes β-galactosidase	*Escherichia coli*
ColE1		ColE1	origin of plasmid replication in *Escherichia coli*	*Escherichia coli*

The *bla* gene

The *bla* gene is derived from *Escherichia coli* and encodes β-lactamase which confers resistance to ampicillin. The *bla* gene is under the control of a bacterial promoter and was included as a marker to allow for selection of bacteria containing pCIB3064 and pCIB4431 prior to transformation of the plant cells. The *bla* gene has no plant regulatory sequences and is unlikely to be expressed in plant tissues.

Characterisation of the genes in the plant

Studies evaluated:

Privalle, L. 1994 Quantification of Cry1A(b) and PAT proteins in *Bt* corn (corn) tissues, whole plants and silage. Performing laboratory: Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Study No CAB-009-94

Molecular characterisation of the integrated DNA present in glufosinate ammonium-tolerant Bt-176 corn was performed using DNA from untransformed corn CG00526 and plasmids pCIB3064 and pCIB4431 as reference material.

Southern blotting experiments confirmed the presence in Bt-176 corn of the *cry1A(b)* (Koziel *et al* 1993), *bar* and *bla* genes. The data indicate that there may be as many as six copies of the *cry1A(b)* and *bla* genes present in Bt-176, and at least two of the *bar* gene (together with the 35S promoter) as determined by the number of hybridising bands in DNA isolated from Bt-176 corn and digested with restriction enzymes which do not cut inside the gene sequence(s). A summary of the genes transferred to Bt-176 corn is shown in Table 3.

The presence of at least one functional copy of each of the *cry1A(b)* genes under the control of the leaf-specific corn PEPC promoter and the pollen-specific promoter respectively was confirmed from the expression of Cry1A(b) protein in these tissues (see Section 3.3 below).

Table 3. Genes transferred to Bt-176 Corn

Gene	Copy number	Function	Source
cry1A(b)	≥5	Gene encoding Cry1A(b) Bt toxin, insect resistance.	*Bacillus thuringiensis*
bar	≥2	Gene encoding PAT protein, phosphinothricin tolerance.	*Streptomyces hygroscopicus*
P-35S (*bar*)	≥2	Direct transcription of *bar* in plant cells.	Cauliflower mosaic virus
bla	≥5	Ampicillin resistance in bacteria.	*Escherichia coli*

Stability of the genetic changes

Studies evaluated:

Privalle, L. 1994 Genetic stability of the modified corn plants: segregation analyses and Southern blots. Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Appendix B to Study No CAB-009-94

The inheritance of the phosphinothricin-tolerant and ECB-protected phenotypes of Bt-176 corn was analysed by backcrossing experiments. In typical studies, 887 plants comprising 12 three-way cross populations, 1273 plants comprising 11 BC1 (backcross) populations and 699 plants comprising 7 F2 populations were evaluated for phosphinothricin tolerance. Similarly, 207 plants from 5 BC1 populations and 899 plants from 13 BC2 populations were evaluated for protection against ECB. The analysis showed a 1:1 phenotypic segregation ratio for phosphinothricin tolerance and ECB tolerance. These data showed that phosphinothricin tolerance and insect protection co-segregate as tightly linked Mendelian traits, suggesting a single site of insertion of the transgenes with a few copies of each gene (Koziel *et al* 1993).

The low number of recombinants identified further suggested a tight linkage of the transgenic traits: of 3240 plants evaluated from 1993 field trials, only 5 (0.15%) were found to exhibit only one of the introduced traits.

Inbred progeny plants also exhibited the same banding pattern over four generations as the original Bt-176 as demonstrated in Southern blots probed with either the *cry1A(b)* or the *bar* sequences. Restriction Fragment Length Polymorphism (RFLP) analysis of phosphinothricin-tolerant corn plants indicated that the active *bar* gene acts as a single locus and maps between probes CG320 and CG378 (with known map locations) of Chromosome 1 of maize. Probing with the *cry1A(b)* gene indicated that it maps to the same locus as that determined for *bar*.

Levels of expression of the Cry1A(b) protein in leaves and pollen of anthesis stage plants, determined by enzyme-linked immunosorbent assay (ELISA), were stable over four successive backcross generations for two different *Bt* corn lines (original transformant CG00526-176 x CG00642, CG00526-176 x CG00554), with no indication of reduced expression. These data are shown in Table 4.

From these data it can be concluded that the introduced genes have been stably integrated into the corn genome and are stably inherited over four generations.

Conclusions regarding the genetic modification

The *cry1A(b)*, *bar* and *bla* genes were transferred to corn via a microprojectile bombardment transformation system resulting in the generation of the ECB-protected and phosphinothricin-tolerant Bt-176 corn. Segregation analyses indicate that the DNA was integrated into the genome of Bt-176 corn as a single and stable insert.

Table 4. Cry1A(b) and PAT expression over four backcross generations of hybrids of CG00526-176 (Bt-176)

Generation		**Cry1A(b) µg/g dry wt**		**PAT µg/g dry wt**
CG00526-176 x[#]	**n**	**Leaves**	**Pollen**	**Leaves**
BC1				
CG00642	8	6.42 (4.36-9.24)	3.43 (2.90-4.01)	lod*
CG00554	2	5.13 (4.70-5.56)	3.82 (3.81-3.84)	lod*
BC2				
CG00642	3	10.24 (8.52-11.11)	4.25 (3.51-4.66)	lod*
CG00554	5	7.81 (4.5-9.1)	5.86 (4.38-7.02)	lod*
BC3				
CG00642	4	8.89 (7.25-12.18)	4.47 (3.27-6.49)	lod*
CG00554	2	6.58 (4.06-10.3)	6.68 (5.46-7.90)	lod*
BC4				
CG00642	3	7.77 (6.61-8.83)	4.45 (3.90-5.51)	lod*
CG00554	5	11.08 (7.0-15.76)	5.23 (4.74-5.63)	lod*

*lod: limit of detection = trace PAT activity was detectable, but below the limit of quantitation (0.75 µg/g dry wt); range shown in parentheses.
[#]CG00526-176 x CG00642 and CG00526-176 x CG00554

GENERAL SAFETY ISSUES

Bt-176 corn is grown in the USA for both domestic use and for export. Bt-176 corn was approved for environmental release and use in human food in the USA in 1995 (USDA 1995, US FDA 1999) in food in Canada in 1996 and 1995 (CFIA 1995, 1996). According to the applicant, grain harvested from Bt-176 corn will enter the food chain only after processing. Processed foods, including imported processed foods may contain genetically modified Bt-176 corn.

The Bt-176 corn has been assessed according to the safety assessment guidelines developed by ANZFA, relating to Group D foods, i.e. plants or animals that contain new or altered genetic material (ANZFA 1999a).

History of use of corn as food

Corn has been cultivated for centuries and is used as a basic food item by people throughout the world (Wright, 1987). The grain is widely used as a feedstuff, although a large part of corn production is also used for human food products, and a wide variety of food products are derived from corn kernels.

Two milling procedures are used for processing of corn, dry milling 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). Human food products derived from dry

milling include corn flakes, corn flour and grits. Corn flakes are produced by a process that involves high temperatures and pressures, grits are prepared by boiling.

The wet milling process for corn 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 at 52°C 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 (May 1987). These treatments would be expected to degrade and remove proteins (May 1987). Oil is produced from wet-milled corn by solvent extraction and heat (120°C, May 1987) and corn oil is considered to be free of protein (Rogers 1990).

According to the applicant, grain harvested from *Bt-176* dent corn varieties will enter the food chain only after processing. There is the potential that the novel traits could be bred into sweet corn varieties, in the future, and therefore could be consumed as fresh, frozen or canned corn.

Nature of novel proteins

Two new proteins are expressed in *Bt*-176 corn: a truncated form of the insecticidal protein Cry1A(b), and phosphinothricin acetyl transferase (PAT). No β-lactamase enzyme expression is expected in Bt-176 corn as the *bla* gene does not have any regulatory sequences that would be recognized in the plant background.

cry1A(b)

The insecticidal δ-endotoxins referred to as Cry proteins are produced by the aerobic, spore-forming soil bacterium *Bacillus thuringiensis* (Bt) (Schnepf *et al* 1998). There are a multitude of Cry proteins, with particular Cry proteins being toxic to only certain insects. 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 (Gill 1995, Rajamohan *et al* 1998). Aggregation of the core toxins results in 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 synthetic *cry1A(b)* gene encodes a Cry1A(b) protein of 648 amino acids with a predicted molecular weight of 65 kD. The predicted amino acid sequence includes the sequence equivalent to that of the natural Cry1A(b) tryptic fragment (607 amino acids, 60 kD).

PAT

S. hygroscopicus (and other *Streptomyces* spp.) produce the tripeptide antibiotic bialaphos (phosphinothricin alanyl alanine) which consists of phosphinothricin (glufosinate ammonium), an analogue of L-glutamic acid, and two alanine residues. The *bar* (bialaphos

antibiotic resistance) gene encodes the enzyme phosphinothricin acetyl transferase (PAT) which breaks down bialaphos thus giving *Streptomyces* protection from the toxicity of the antibiotic it produces (Thompson *et al* 1987, Kumada *et al* 1988).

Phosphinothricin is used as a broad spectrum herbicide and is a potent inhibitor of glutamine synthetase (GS), the key enzyme in ammonia metabolism in plants. Phosphinothricin application to plants results in a rapid (< 2 hours) increase in the level of free ammonium resulting in cell death (De Block *et al* 1987). The *bar* gene, derived from either *S. hygroscopicus* or *S. viridochromogenes*, has been transferred to a number of plant species other than corn, including tobacco (De Block *et al* 1987, Wohlleben *et a*l 1988), canola (Beriault *et al* 1999), sugar cane (Gallo-Meagher and Irvine 1996) and rice (Cao *et al* 1992) to confer tolerance to glufosinate ammonium.

Expression of novel protein in the plant

Studies evaluated:

Privalle, L. 1994 Characterisation of Cry1A(b) protein produced in *Bt* corn (corn) Event 176 and comparison with native Cry1A(b) protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD1-9. Performing laboratory: Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Study No CAB-006-94

Privalle L. 1994 Quantification of Cry1A(b) and PAT proteins in *Bt* corn (corn) tissues, whole plants and silage. Performing laboratory: Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Study No CAB-009-94

Ciba Seeds petition for the Determination of Nonregulated Status for Insect-Resistant Event 176 Corn. Submitted to the USDA/APHIS on November 15, 1994. Reference number 94-319-01. Chapter 6.

The expression of Cry1A(b) and PAT in corn plants derived from *Bt*- 176 corn was determined for various plant tissues and developmental stages in three corn lines from field tests carried out in the United States in 1993, and in selected tissues of mature greenhouse-grown inbred plants representing four additional genotypes and four backcross generations of two genotypes.

Cry1A(b)

The presence of Cry1A(b) protein in leaves and pollen of *Bt*-176 corn was demonstrated by Western blotting with an antibody specific to the native Cry1A(b) protein. There were two major immuno-reactive protein bands with apparent molecular weights of approximately 65 kD, as predicted from the *cry1A(b)* gene sequence (see Section 3.2 above). The bands correspond to the predicted sizes of the 648 amino acid truncated Cry1A(b) protein with and without the first 24-28 amino acids. Three other immuno-reactive bands of lower molecular weight were observed in Western blots of leaf extracts: 60kD; 40kD; and 36kD. The tryptic fragment of the Cry1A(b) protein has a molecular weight of 60kD. It was demonstrated that the presence of these bands of less than 65kD were not artefacts of the extraction procedure.

The 65kD and 36kD immunoreactive protein bands from leaf tissue were subjected to N-terminal amino acid sequencing. The N-terminus of the 65kD band corresponded with amino acid 25 of the predicted sequence and the sequence was identical to that expected over the 10 amino acids sequenced. The N-terminus of the 36kD protein corresponded with amino acid 31 of the predicted sequence and the sequence was identical to that expected over the 15

amino acids sequenced. This identified the 36kD protein as the N-terminal fragment that extends to amino acid 350-400 as encoded by the native and synthetic cry1A(b) genes. From these data the applicant reasoned that the N-terminal processing of the 65kD protein, and the bands of less than 65kD, resulted from the action of intrinsic corn proteases rather than the presence of truncated gene sequences.

Immuno-purified Cry1A(b) protein from Bt-176 corn was tested for post-translational modifications. No evidence was found of acetylation, glycosylation or phosphorylation. Additionally, the functionality of the Cry1A(b) protein expressed in Bt-176 corn and hybrids was demonstrated by resistance to attack by ECB. The Cry1A(b) immunoreactive proteins of less than 60kD are not expected to contribute to insecticidal activity as they are below the minimum size needed for bioactivity.

The Cry1A(b) protein was detected by ELISA in significant quantities in leaves and pollen, as expected given the tissue specificity of the promoters (Table 5a). Trace amounts of the Cry1A(b) protein were detected in kernels as well as in other plant tissues (roots and pith) but were below the limit of quantification. Whole plants selected at various stages throughout the growing season were assessed for their level of Cry1A(b) protein which was measured to be highest in plants (per gram dry weight) selected at seedling stage and decreased during the rest of the growing season (Table 5a).

When considered as a proportion of total plant protein, the highest mean level of Cry1A(b) protein was measured in whole plants - 14.4 μg/g total protein. This was observed in homozygous *Bt*-176 corn (inbred) plants taken at anthesis. This represents 0.00144% of the total protein. Trace levels of Cry1A(b) protein were detected in fresh kernels, however the levels were below the limit of quantification of 5 ng/g fresh weight kernels (5 ppb). Consistent results have been determined in several genetic backgrounds including hybrids 176 x 554 and 176 x 564 (Tables 5a and 5b).

The presence of the Cry1A(b) protein in fresh kernels was verified by a bioassay of insecticidal activity against ECB larvae. There was no significant insecticidal activity against ECB in dried and re-hydrated kernels. The data are shown in Table 6.

PAT protein levels

Expression of functional PAT protein in Bt-176 corn was evidenced by increased tolerance to phosphinothricin (glufosinate ammonium herbicide). PAT was detected by ELISA in trace quantities in leaves, roots, pith and whole plants, but the levels were below the limit of quantification (Table 5a). No PAT was detected in either kernels or pollen. The level of PAT expressed in Bt-176 corn was at least thirty times less than in phosphinothricin-tolerant corn lines developed by Novartis for agronomic use, and Bt-176 corn is not intended for use as a herbicide-resistant plant.

β-lactamase

The *bla* gene introduced into Bt-176 corn is under the control of a bacterial promoter and would not be expected to be expressed in plant tissues. However, expression of the *bla* gene in Bt-176 corn was investigated by assay of β-lactamase activity and Northern blotting. No β-lactamase activity was detected in protein extracts of either leaves or pollen of Bt-176 corn. Northern blotting of total RNA from leaves of Bt-176 corn did not detect any *bla* mRNA transcripts. These results confirm, as predicted, that there is no β-lactamase expression in Bt-176 corn.

Table 5a: Cry1A(b) and PAT Protein levels in Bt176 corn and hybrid lines during development.

	Stage of development µg/g dry weight (n)							
	Seedling		**Anthesis**		**Seed Maturity**		**Senescence**	
Leaves	Cry1A(b)	PAT	Cry1A(b)	PAT	Cry1A(b)	PAT	Cry1A(b)	PAT
Bt176[1]	10.5 (3)	<1.5	3.04 (3)	<0.75	1.43 (3)	<0.40	0.10 (2)	nd
176 x 554[2]	4.78 (5)	<1.5	2.70 (3)	nd	1.65 (3)	nd	0.12 (3)	nd
176 x 564[3]	7.56 (3)	<1.5	13.37 (2)	nd	1.52 (2)	<0.40	0.30 (3)	<0.30
Whole Plant								
Bt176	4.19 (3)	nd	1.44 (5)	<1.20	0.29 (4)	<0.50	<0.02 (5)	<0.35
176 x 554	2.85 (6)	<2.30	0.20 (3)	nd	0.15 (4)	<0.50	<0.02 (4)	<0.35
176 x 564	3.40 (2)	<2.30	0.74 (3)	nd	0.26 (4)	nd	<0.02 (3)	<0.35
Kernels								
Bt176	---	---	---	---	<0.01 (4)	nd	<0.01 (5)	nd
176 x 554	---	---	---	---	<0.01 (3)	nd	<0.01 (3)	nd
176 x 564	---	---	---	---	<0.01 (2)	nd	<0.01 (3)	nd
Pollen[4]								
Bt176	---	---	4.32 (4)	nd	---	---	---	---
176 x 554	---	---	2.34 (3)	na	---	---	---	---
176 x 564	---	---	5.01 (3)	nd	---	---	---	---
Roots								
Bt176	<0.1 (2)	nd	<0.04 (4)	<0.90	<0.04 (4)	<0.90	na	na
176 x 554	<0.1 (6)	nd	<0.04 (3)	<0.90	<0.04 (3)	<0.90	na	na
176 x 564	<0.1 (1)	nd	<0.04 (3)	nd	<0.04 (2)	<0.90	na	na
Pith								
Bt176	na	na	<0.07 (4)	<1.60	<0.04 (4)	<0.85	na	na
176 x 554	na	na	<0.07 (3)	nd	<0.04 (3)	<0.85	na	na
176 x 564	na	na	<0.07 (3)	na	<0.04 (2)	nd	na	na

---: not relevant at this development stage; na: not analysed; nd: not detectable = the mean ELISA absorbance did not exceed that of the control equating to 0 ng protein;
[1]Genotype Bt176 refers to the genetically modified corn line CG00526-176 which is homozygous for both the *cry1A(b)* and *bar* genes;
[2]Genotype 176 x 554 refers to the hybrid corn line developed by the cross of CG00526-176 and untransformed line CG00554 and is hemizygous for the introduced genes.
[3]Genotype 176 x 564 refers to the hybrid corn line developed by the cross of CG00526-176 and untransformed line CG00564 and is hemizygous for the introduced genes
[4]Pollen values were determined on dry pollen samples and extrapolated to fresh weight.

Table 5b: Cry1A(b) levels in Bt-176 corn hybrids[1]

Line	**Kernels** at seed maturity	**Whole plants** at seed maturity	**Whole plants** at anthesis
µg Cry1A(b)/g total protein[2]			
526	0	0	0
Bt-176	<0.09[3]	3.63	14.40
526 x 554	0	0	0
Bt-176 x 554	<0.09[3]	2.14	2.50
526 x 564	0	0	0
Bt-176 x 564	<0.10[3]	3.71	7.40
Cry1A(b) as % total protein			
526	0	0	0
Bt-176	0.000009%	0.000363%	0.00144%
526 x 554	0	0	0
Bt-176 x 554	0.000009%	0.000214%	0.00025%
526 x 564	0	0	0
Bt-176 x 564	0.00001%	0.000371%	0.00074%

[1]Bt-176 = CG00526-176 homozygous inbred line, 554 = CG00554, 564 = CG00564, 526 = CG00526.
[2]µg/g protein values derived by calculation from values in Table 5a, plants grown in 1993 field trials in Hawaii
[3]Below the limit of quantification, --: not determined,

Table 6. Presence of Cry1A(b) protein in kernels: bioassay of insecticidal activity on European Corn Borer larvae.

Kernels	**% mortality** mean ± standard deviation
Fresh kernels	76.5 ± 10.9
Dry kernels	13.3 ± 4.7
Rehydrated kernels	3.3 ± 4.7

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

Studies evaluated:

Duck, N. and Peters, C. 1995 Attempts to select ampicillin-resistant E. coli by transformation with DNA from the genetically modified maize. Performing laboratory: Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA.

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 Expert Consultation which looked at strategies for assessing the safety of foods produced by biotechnology (WHO 1991). It was concluded by that consultation 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.

This section of the report will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from insect-protected corn to microorganisms present in the human digestive tract.

The two plasmids used to transform corn line CG00526 - pCIB4331 and pCIB3064 - both contained a copy of the *bla* gene under the control of a bacterial promoter. The *bla* gene encodes the enzyme β-lactamase and confers resistance to a number of β-lactam antibiotics such as penicillin and ampicillin. Analysis of the Bt-176 corn and its hybrids confirmed the presence of as many as six intact copies of the *bla* gene along with its bacterial promoter. The *bla* gene is not expected to be expressed in the Bt-176 corn lines because it is under the control of a bacterial promoter and lacks regulatory sequences that would be recognized in plants. Experimental evidence discussed in Section 3.3 demonstrated no expression of the *bla* gene, as expected.

Potential for horizontal gene transfer

The first issue that must be considered in relation to the presence of an intact *bla* gene in Bt-176 corn is the probability that this gene would be successfully transferred to and expressed in microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

- excision of DNA fragments containing the *bla* gene and its bacterial promoter;
- survival of DNA fragments containing the *bla* gene in the digestive tract;
- natural transformation of bacteria inhabiting the digestive tract;
- survival of the bacterial restriction system by the DNA fragment containing the *bla* gene;
- stable integration of the DNA fragment containing the *bla* gene into the bacterial chromosome or plasmid; and
- maintenance and expression of *bla* gene by the bacteria.

[1] Food and Agriculture Organization.

The transfer of a functional *bla* gene to microorganisms in the human digestive tract is therefore highly unlikely because of the number and complexity of the steps that would need to take place consecutively.

The second and most important issue that must be considered is the potential impact on human health in the unlikely event successful transfer of a functional *bla* gene to microorganisms in the human digestive tract did occur.

In the case of transfer of the *bla* gene from Bt-176 corn to microorganisms of the digestive tract, the human health impacts are considered to be negligible. This is because ampicillin-resistant bacteria are commonly found in the digestive tract of healthy individuals (Calva *et al* 1996) as well as diseased patients (Neu 1992). Therefore, the additive effect of a *bla* gene from Bt-176 corn being taken up and expressed by microorganisms of the human digestive tract would be insignificant compared to the population of ampicillin resistant bacteria already naturally present.

The transfer of novel genetic material from genetically modified food to human cells via the digestive tract is also 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 special additional risks compared with the large amount of DNA naturally present in all foods.

The applicant assessed the possibility of transfer of the *bla* gene from Bt-176 corn to bacteria by attempts to transfer ampicillin resistance through transformation of *E. coli* bacteria with total DNA extracted from Bt-176 corn plants. No ampicillin resistant colonies were isolated from either transformationally competent or non-competent *E. coli* cells treated with either intact or degraded DNA from Bt-176 corn.

The processing steps for corn typically include heat, solvent or acid treatments that would be expected to remove and destroy DNA. Intact fragments of the *bla* gene are unlikely to survive the processing steps making the chance of horizontal gene transfer even more unlikely. The processing steps can also lead to the release of cellular enzymes (nucleases) which are responsible for degrading DNA into smaller fragments.

Other relevant data

Studies evaluated:

Privalle, L. 1994 Assessment of DIMBOA levels in transgenic *Bt* corn (corn) and nontransgenic corn. Ciba Seeds, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Report No CAB-015-94.

To determine whether the transformation process had caused unintended changes in endogenous corn gene expression, levels of DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one), were measured in transgenic *Bt* corn and compared to levels in isogenic control plants. DIMBOA is a natural plant defence compound (Gierl and Frey 1999) and has been correlated with natural resistance to ECB larvae (Klun and Brindley 1966).

Analyses were conducted by HPLC after conversion of DIMBOA to the more stable MBOA (6-methoxy-2(3*H*)-bezoxazolone). The data are shown in Table 7.

No significant differences were observed between transgenic and control plants, although considerable plant to plant variation was observed. The data support the conclusion that the introduction of the Cry1A(b) gene has not resulted in any unintended perturbation of endogenous gene expression related to natural plant defence mechanisms.

Table 7. Levels of MBOA in Bt-176 corn leaves

Genotype	**N**	**MBOA** μg/g fr wt ± standard deviation
Control CG00526 inbred	10	0.86 ± 0.38
CG00526-Bt 176 inbred	10	0.84 ± 0.36

Conclusions regarding general safety issues

The *cry1A(b)* and *bar* genes are expressed in insect-protected corn containing the Bt-176 transformation event. The Cry1A(b) protein is expressed at the highest levels in leaves and pollen, as expected from the tissue specificity of the PEP-C and P-pollen promoters, but below the level of quantification in the kernel. The PAT protein is also expressed in trace amounts in all tissues except kernels, where it was undetectable. The levels of protein and DNA in highly processed corn products such as corn oil, high fructose corn syrup and corn starch is considered negligible, and therefore the level of Cry1A(b) and PAT genes and proteins would be vanishingly small. The Cry1A(b) and PAT genes and proteins have been well characterised. The transfer of these genes to corn is not considered to be a risk public health and safety.

It is extremely unlikely that the ampicillin resistance gene will transfer from foods derived from insect-protected Bt-176 corn to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. In the highly unlikely event that the resistance gene was transferred to bacteria in the human digestive tract the human health impacts would be negligible because ampicillin-resistant bacteria are already commonly found in the human gut and in the environment.

It is also equally unlikely that novel genetic material from the insect-protected Bt-176 corn will be transferred to human cells via the digestive tract. The novel genetic material comprises only a minute fraction of the total DNA in the insect-protected Bt-176 corn therefore it is unlikely to pose any special additional risks compared with the large amount of DNA naturally present in all foods.

The probable degradation and removal of DNA through the processing steps for corn further mitigate against any horizontal transfer of DNA from insect-protected Bt-176 corn to cells in the human digestive tract.

TOXICOLOGICAL ISSUES

Levels of naturally-occurring toxins

There are no naturally occurring toxins known to occur at biologically significant levels in corn (Wright, 1987).

Potential toxicity of novel proteins

Studies evaluated:

Kuhn, J.O. 1994a Cry1A(b) *B.t.k.* delta-endotoxin. Acute oral toxicity study in mice. Performing Laboratory: Stillmeadow Inc, Sugar Land, Texas. Study No 1238-94Sponsor: Ciba-Geigy Corporation, Research Triangle Park, NC, USA.

Kuhn, J.O. 1994b *Bt* Corn Leaf Protein Lot: LP176-0194 and Control Corn Leaf Protein Lot: LP176-0194C. Acute oral toxicity study in mice. Performing Laboratory: Stillmeadow Inc, Sugar Land, Texas. Study No 1443-94 Sponsor: Ciba-Geigy Corporation, Research Triangle Park, NC, USA.

Campbell, S.M. 1994 Cry1A(b) enriched corn leaf protein: An acute oral toxicity study with the northern bobwhite (*Colinus virginianus)*. Performing lab. Wildlife International Ltd. Project No 108-371 Sponsor: Ciba Seeds, Research Triangle Park, NC, USA.

Kuhn, J.O. 1995 Phosphinothricin acetyltransferase (Sample PAT-0195) Acute oral toxicity study in mice. Performing Laboratory: Stillmeadow Inc, Sugar Land, Texas. Study No 1910-95 Sponsor: Ciba-Geigy Corporation, Research Triangle Park, NC, USA

Privalle, L. 1994 Characterisation of Cry1A(b) protein produced in *Bt* corn (corn) Event 176 and comparison with native Cry1A(b) protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD1-9. Performing laboratory: Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Study No CAB-006-94

Neither of the newly expressed proteins was found to have any similarity to a database of 2632 sequences of known toxins. The potential toxicity of the Cry1A(b) and PAT proteins was assessed by Novartis by evaluating acute oral toxicity in mice and in birds. The scientific basis for using an acute test is that known protein toxins generally act via acute mechanisms (Jones and Maryanski 1991).

Novartis carried out four acute toxicity studies: three in mice, using corn-expressed Cry1A(b), native Cry1A(b) and PAT; and one in the northern bobwhite quail using corn-expressed Cry1A(b).

Cry1A(b)

Cry proteins from *B. thuringiensis* have a long history of safe use as insecticides. There is no evidence from this history of use that there is any associated toxicity to humans. The toxicity of these proteins is very specific to Lepidopteran insects and there is no evidence that they are active against non–target insects, birds, fish or mammals (Hadley *et al* 1987, Drummond and Pinnock 1991). This 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 specific gut receptors in other organisms (Frick 1995). The binding of the δ–endotoxin to specific gut receptors appears to be a pre–requisite for toxicity (Cooper 1991, Schnepf *et a*l 1998). *In vivo* studies with rats given Cry1A(b) orally, and *in vitro* studies with rats, mice, rhesus monkeys and humans did not reveal receptors for the protein (Noteborn *et al* 1995).

(i) Equivalence of the plant Cry1A(b) protein to the native protein.

Cry1A(b) protein derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD1-9 as well as plant derived protein was used in acute toxicity studies. Characterisation of the Cry1A(b) proteins from Bt-176 corn and from *B. thuringiensis* confirmed that the proteins were comparable in physical and chemical properties (see Section 3.2 above).

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

Cry1A(b) δ-endotoxin purified from *Bacillus thuringiensis* (purity 70%) was administered to the mice (5/sex) at 5050 mg total protein/kg body weight by single oral gavage. There were no adverse effects from the dosing volume.

Mice were observed for clinical signs at least 3 times on the day of dosing and once daily for 14 days. 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.

No deaths occurred during the study. The only abnormal clinical sign observed was piloerection (raised hair), which occurred only on day 1. During the second week after dosing, one female lost weight; all other mice showed normal body weight gains for their age and sex. No abnormalities were detected on necropsy. Taking account of the purity of the protein preparation, the acute oral LD_{50} for Cry1A(b) δ-endotoxin was therefore concluded to be >3535 mg/kg bw in mice.

(iii) Acute oral toxicity in mice – Bt-176 Cry1A(b)

A protein extract from Bt-176 corn leaves enriched for Cry1A(b) (0.07%) or control corn leaf protein was administered to the mice (5/sex) at a dose of 5050 mg leaf protein /kg bw by single oral gavage, corresponding to a dose of plant-derived Cry1A(b) protein of 3.54 mg/kg bw.

Mice were observed for clinical signs at least 3 times on the day of dosing and once daily for 14 days. 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.

Two animals that received the test material died; however one of the deaths (male on day 1) was caused by a dosing injury. One animal that received the control material died (female on day 1). Gross necropsy of all animals at the end of the study revealed abnormalities only in those animals that died: in the test animal that died on day 1, there was perforation of the oesophagus, in the test animal that died on day 2, there were abnormalities of the lungs and liver and in the control animal that died on day 1, there were lung and stomach content abnormalities.

There were no significant differences in clinical findings or body weight gain between the group receiving leaf protein containing Cry1A(b) protein and that receiving control leaf protein. Clinical signs in the test group included piloerection, lacrimation (crying), polyuria, ptosis and decreased activity. Except for piloerection, these were seen at a low frequency, and all clinical signs had resolved by day 4. In the control group, piloerection and decreased activity were seen until day 5. One female dosed with the test material, and two females

dosed with the control material lost weight during the second week of the study; bodyweight in the remaining animals was considered normal for their age and sex. On gross post-mortem examination, there were no treatment-related abnormalities.

The applicant concluded that it could not be categorically ruled out that a maize protein component present in both the control and Cry1A(b) protein preparations was responsible for the mortality observed in this study. On the basis of the data presented, ANZFA concurs with this conclusion. These mortalities do not appear to be due to the Cry1A(b) protein as the female mice were from both the test and control groups thus suggesting some other component of the leaf extract. It should also be noted that there was no incremental increase in mortality throughout the study as might be expected if the observed deaths were due to a component of the corn leaf extracts. The acute oral LD_{50} of Bt-176 corn leaf protein was therefore determined to be > 5050 mg/kg bw in mice, corresponding to 3.54 mg Cry1A(b) protein /kg bw.

(iv) Acute oral toxicity in birds – Bt-176 Cry1A(b)

Bt-176 corn leaf protein (0.07% Cry1A(b)) or control leaf protein was administered to 8 week old Northern bobwhite quail (5/sex) at 2000 mg protein/kg bw by single oral gavage corresponding to a dose of plant-derived Cry1A(b) protein of 1.4 mg/kg bw.

Following dosing, all birds were observed at least twice daily for mortality, signs of toxicity or abnormal behaviour. Bodyweight was measured one to two days before dosing and on days 3, 7 and 14. Average feed consumption was determined for each group for days 0–3, 4–7 and 8–14. At the end of the observation period the birds were killed and post-mortem examinations conducted.

No birds died during the test period and there were no abnormal clinical signs or behavioural changes in any group. There were no treatment-related effects on bodyweight or food consumption during this study and no abnormalities were detected on post-mortem examination. The acute oral LD_{50} for northern bobwhite quail exposed to modified corn leaf protein (0.07% Cry1A(b) protein) was therefore concluded to be >2000 mg protein/kg bw, corresponding to 1.4 mg Cry1A(b) protein /kg bw.

PAT protein

An exemption from requirement to establish a maximum permissible level for residues of PAT and the genetic material necessary for its production was granted by the United States Environmental Protection Agency in April 1997 (US EPA 1997). Data demonstrating the absence of acute oral toxicity of PAT in mice have been evaluated by ANZFA for another application (Application A380 DBT-418 corn, Merriman 1996).

The PAT protein was expressed in trace amounts in Bt-176 corn but was at the limit of quantitation (see 3.2 above). This level of PAT expression would have been insufficient to allow extraction of adequate quantities for use in toxicity or digestive lability experiments. PAT protein was therefore derived from expression of the recombinant protein in *E. coli*.

Groups (5/sex) of mice were given a single oral dose (gavage) of either PAT protein (purity 51%) in carboxymethyl cellulose; heat inactivated PAT (52% purity) in carboxymethyl

cellulose; or carboxymethyl cellulose control to a total dose of 5050 protein mg/kg bw, or adjusted for purity, 2575 mg PAT protein/kg bw.

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 as a result of material lodged in the oesophagus. The globule of solid material was sufficient to prevent passage of food or water into the stomach and is the likely cause of death of this animal. The only notable clinical signs were decreased activity, piloerection and ptosis on days 6–8 in the male that died. One male receiving the reference substance showed slight piloerection on the day of dosing. 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 acute oral LD_{50} of PAT protein was concluded to be >2575 mg/kg bw.

Potential allergenicity of existing proteins

There are no naturally occurring allergenic proteins known to occur in corn (Wright, 1987).

Potential allergenicity of novel proteins

Studies evaluated:

Privalle, L. 1994 Characterisation of Cry1A(b) protein produced in *Bt* corn (corn) Event 176 and comparison with native Cry1A(b) protein produced by *Bacillus thuringiensis* subsp *kurstaki* strain HD1-9. Ciba Seeds Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Report No. CAB-006-94

Privalle, L 1994 *In vitro* digestibility of CryIA (b) protein from Bt corn (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. Study No. CAB-007-94

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. Study No. CAB-008-94.

Although there are no predictive assays available to definitively assess the allergic potential of proteins, a number of characteristics are common among many of the allergens that have been characterized (Lehrer and Reese 1998, Jones and Maryanski 1991). Known allergens tend to be glycosylated proteins with a molecular weight of 10–70 kD (Lehrer *et al* 1996**)**. Protein 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). 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).

The Cry1A(b) and PAT proteins were evaluated for potential allergenicity against these criteria: size; glycosylation; resistance to heat (PAT), digestive degradation and sequence similarity to known allergens.

Cry1A(b) protein

The *cry1A(b)* gene was derived from *B. thuringiensis* subsp. *kurstaki*. *B. thuringiensis* is not a food source but is a common soil bacterium that may be found on or around plant produce. Cry proteins have been used extensively as insecticides for decades and there are no reports of allergic reactions from either occupational exposure or ingestion of fresh produced sprayed with such insecticides.

The molecular weight of the Cry1A(b) protein expressed in Bt-176 corn is 65 kD, and thus within the size range of typical allergens. As described above (Sections 2.1 and 3.3) the synthetic cry1A(b) gene encodes a protein identical to that of the native tryptic fragment, and the N-terminal amino acid sequence of the Cry1A(b) protein produced in Bt-176 corn was determined to match that of the native protein. The Cry1A(b) protein sequence contains six potential N-glycosylation sites. Direct testing of Cry1A(b) immunopurified from Bt-176 corn was negative for glycosylation and acetylation. Direct testing for phosphorylation of the native tryptic fragment of Cry1A(b) from *B. thuringiensis* was also negative. Given the equivalence of the corn-produced and native Cry1A(b) proteins it is also unlikely that the Cry1A(b) protein in Bt-176 corn is phosphorylated. Western blots indicated that the relative mobility of Cry1A(b) protein from Bt-176 corn did not differ from the predicted molecular weight. These data support the conclusion that the Cry1A(b) is not subject to post-translational modification *in planta*.

(i) Digestibility of Cry1A(b) protein under simulated gastric conditions

The digestibility of Cry1A(b) protein obtained from both genetically modified corn and from *Bacillus thuringiensis* subsp *kurstaki* (*Btk*) was assessed in simulated gastric conditions. Both proteins yield the same active fraction following proteolytic cleavage in the alkaline gut of Lepidopteran insects.

The digestive lability of Cry1A(b) protein extracted from leaves of mature field grown hybrid plants of Bt-176 corn and native Cry1A(b) protein extracted from *Bacillus thuringiensis* subsp *kurstaki* strain HDI-9 was assessed in simulated gastric fluid (SGF, 3.2 mg/ml pepsin at 1x, 0.1x, 0.01x and 0.001x).

The Cry1A(b) protein derived from Bt-176 corn was rapidly degraded in 1x SGF such that no immunoreactive Cry1A(b) polypeptides were detectable by Western blot upon immediate sampling and was undetectable after 10 minutes incubation with 0.001x SGF. The native Cry1A(b) protein was almost all degraded after 2 minutes in 1x SGF and was undetectable after 5 minutes with 0.01X SGF.

These data demonstrate that the Cry1A(b) protein expressed in Bt-176 corn and its hybrids is rapidly degraded in simulated digestive conditions. These results are consistent with published studies (Noteborn *et al* 1995, Sanders *et al* 1998).

Comparisons of the Cry1A(b) protein sequence from *B. thuringiensis* subsp *kurstaki* against sequences present in public domain databases (GenBank, EMBL, PIR and SwissProt) by

Monsanto Pty Ltd, as part of Application A346 for Bt corn MON810, revealed no biologically significant homology with sequences other than Bt insecticidal proteins (Astwood 1995).

PAT protein

The *bar* gene encoding PAT was derived from *Streptomyces hygroscopicus*. The PAT protein is not found in plants or animals and is therefore not a normal component of food. However *Streptomyces* is a common soil bacterium which may be found on and around plant produce.

(i) Digestibility of PAT protein under simulated gastric conditions

The digestive lability of PAT protein was assessed in simulated gastric fluid (SGF, 3.2 mg/ml pepsin at 1x, 0.1x, 0.01x and 0.001x). The presence of PAT in the fluid following incubation was determined by SDS-PAGE analysis. The PAT activity was also determined after incubation in SGF at the pH optimum for the enzyme, at gastric pH and following serial incubation with a gastric solution containing 0.0032 mg/ml pepsin.

The PAT protein was rapidly degraded in 1x SGF such that no polypeptides were detectable by SDS-PAGE upon immediate sampling and was undetectable after 2 minutes incubation in 0.01x SGF. The apparent half-life of PAT in 0.001x SGF was between 1 and 2 minutes.

Incubation of PAT protein at 37°C for 10 minutes resulted in a 44% loss in activity. The heat sensitivity of PAT to temperatures above 35°C has previously been reported (Botterman *et al* 1991). PAT activity was not detected after 1 minute incubation in SGF without pepsin (pH1.0). Activity was not restored by neutralisation.

The amino acid sequence of the PAT protein was compared to the amino acid sequences of known allergens present in the GenBank public domain databases. No biologically significant homology was found with any known allergens or toxins.

These data indicate that the PAT protein will be destroyed upon exposure to the temperature, acid and peptidases of the mammalian gastric system and therefore is unlikely to act as an allergen.

Other relevant data

(i) Residues of glufosinate or its metabolites

Glufosinate is a herbicide commonly used on crops in the USA. No maximum residue limits have been set for glufosinate in grain crops in Australia (Standard A14 – Maximum Residue Limits, ANZFA 1999b). Glufosinate is not considered to be toxic to mammals at the levels applied in agriculture (Ebert *et al* 1990, Hack *et al* 1994), although ingestion of large amounts of the herbicide can result in severe pathology including neurological effects (Watanabe and Sano 1998).

However Bt-176 corn is not intended for use as a herbicide-resistant crop because the very low level of PAT expression (see Section 3.3 above) is insufficient to confer resistance to

commercial doses of glufosinate ammonium. As there will be no herbicide application there will be no glufosinate residues present in Bt-176 corn.

Conclusions regarding toxicological issues

In all studies the acute oral toxicity of Cry1A(b) and PAT proteins was low. In mice the LD_{50} of the native Cry1A(b) protein was >3535 mg/kg bodyweight. The LD_{50} of Cry1A(b)-containing leaf extracts of Bt-176 corn was >5050 mg/kg bodyweight in mice and >2000 mg/kg bodyweight in the northern bobwhite quail. 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). The LD_{50} of PAT in mice was >2575 mg/kg bodyweight.
The data and analyses on the potential for toxicity or allergenicity of the Cry1A(b) or PAT proteins support the conclusions that neither protein is derived from an allergenic or toxic food source nor exhibits the characteristics of known protein allergens. Neither protein exhibits sequence similarity with known toxins or allergens. Furthermore, the Cry1A(b) and PAT proteins are present at very low abundance in corn kernels and both have been shown to be degraded in conditions that mimic human digestion. In addition, the activity of the PAT protein was shown to be destroyed by temperatures in excess of 37°C and by acid pH that would be encountered in the digestive system.

From these data it can be concluded that the food products derived from insect-protected Bt-176 corn should pose no greater threat as a source of allergic reaction than food products from conventional corn.

NUTRITIONAL ISSUES

Studies evaluated:

Privalle, L. 1994 Compositional analysis of kernels from transgenic *Bt* corn (corn) as compared with nontransgenic control corn. Ciba Seeds, Ciba-Geigy Corporation, Research Triangle Park, NC, USA. Report No CAB-016-94.

Compositional analysis

Corn used for compositional analyses was derived from field trial and glasshouse experiments conducted in 1993 and 1994 (see Table 8). A range of analyses were performed on grain of Bt-176 corn and hybrids containing the Bt-176 transformation event. The components measured included proximates (protein, fat, ash, starch, moisture, fibre), amino acid composition, fatty acids profile and carotenoids. In the Hawaiian field trial, five random 100g samples of kernels of each genotype were taken from a pooled sample representing multiple plants. These samples were used for all analyses except fatty acids. One analysis was done on each of the five samples. In the French field trial, two 12.5g samples of kernels of each genotype were taken from a pooled sample representing multiple plants. These samples were used for all analyses except fatty and amino acids. Two analyses were done on each of the two samples. Genetically modified inbred and hybrid lines were compared to their corresponding non-genetically modified controls by t-tests. Significance was judged at the level of $p = 0.05$.

All analyses of the genetically modified and control corn kernels were conducted by Southern Testing and Research Laboratories Inc. (Wilson, NC) using recognised published methods in accordance with either the Association of Official Analytical Chemists (AOAC), the

American Association of Cereal Chemists (AACC) or the American Oil Chemists Society (AOCS).

(i) Proximate analysis

The proximate analyses of kernels from corn lines containing the Bt-176 transformation event did not vary markedly from the isogenic control lines, suggesting that there were no unintentional changes to grain composition due to the genetic transformation. However some statistically significant differences were observed (5% level using a pairwise T test). The data are shown in Tables 9a and 9b.

The protein levels of some hybrid Bt-176 lines varied from the isogenic controls: CG00554 x CG00526-176 (9% lower); CG00637 x CG00526-176 (12% higher); CG00615-176 inbred (17% higher). All the protein levels observed for the various lines, both modified and unmodified, were comparable to values reported by Watson (1987) and Wright (1987).

Table 8. Inbred and hybrid lines of Bt-176 corn used in compositional analyses

	Genotype	**Location**	**Harvest**
Control Bt	inbred CG00526 inbred CG00526-176	field, Hawaii, USA	1993
Control Bt	hybrid CG00554 x CG00526 hybrid CG00554 x CG00526-176	field, Hawaii, USA	1993
Control Bt	hybrid CG00637 x CG00526 hybrid CG00637 x CG00526-176	field, Hawaii, USA	1993
Control Bt	hybrid CG00684 x CG00526 hybrid CG00684 x CG00526-176	field, Hawaii, USA	1993
Control Bt	inbred CG00615 inbred CG00615-176	field, France	1994
Control Bt	hybrid CG00635 x XG00615 hybrid CG00635 x XG00615-176	glasshouse, France	1994

The fat levels of some hybrid Bt-176 lines varied from the isogenic controls: CG00554 x CG00526-176 (65% higher); and CG00684 x CG00526-176 (44% less). Fat levels for all lines were comparable to reported values (Watson 1987) and the fat content was less than 5% of kernel in all cases.

The starch level of the hybrid CG00684 x CG00526-176 was 17% higher than the CG00684 x CG00526 control. The moisture content of some hybrid lines varied from the isogenic controls: CG00554 x CG00526-176 (27% higher); and CG00684 x CG00526-176 (26% less).

It should also be noted that there was considerable variability between lines for all of the components tested and that the differences observed in some lines were not evident in all lines, suggesting that the differences are not a result of the genetic modification.

Table 9a. Compositional analysis of kernels from inbred CG00526-176 Corn[1]

Component	Control CG00526	Bt CG00526-176	Literature Range[2](%)
Protein %	12.21 ± 0.43 (11.60-12.74)	11.71 ± 0.35 (11.23-12.05)	6-12
Total fat %	4.74 ± 0.80 (3.84-5.65)	4.07 ± 0.80 (3.30-5.38)	3.1-5.7
Ash %	1.44 ± 0.03 (1.38-1.46)	1.41 ± 0.04 (1.35-1.45)	1.1-3.9
Starch %	65.85 ± 4.17 (60.86-72.08)	69.05 ± 0.88 (68.69-70.84)	65.3-83
Fibre %	1.95 ± 0.08 (1.85-2.05)	1.86 ± 0.13 (1.69-2.03)	2.5[3]
Moisture %	11.94 ± 0.44	12.38 ± 0.34	7-23

[1]n=5, Mean ± standard deviation (range), plants from 1993 field trials in Hawaii
[2]Watson 1987
[3]average value

Table 9b. Proximate analysis of hybrid and inbred lines containing the Bt-176 transformation event, % component

Genotype	n	Protein	Fat	Ash	*Starch*	*Fibre*	Moisture
Control 526	5	12.21 ± 0.43	4.74 ± 0.80	1.44 ± 0.03	65.85 ± 4.17	1.95 ± 0.08	11.94 ± 0.44
526-Bt 176	5	11.71 ± 0.35	4.07 ± 0.80	1.41 ± 0.04	69.95 + 0.88	1.86 ± 0.13	12.38 ± 0.34
Control 554x526	5	11.96 ± 0.35	2.55 ± 1.14	1.30 ± 0.05	68.29 ± 10.06	1.50 ± 0.13	9.64 ± 0.40
554xBt-176 hybrid	5	***10.88 ± 0.17****	***4.21 ± 0.79****	1.27 ± 0.03	72.19 ± 2.56	1.41 ± 0.12	***12.23 ± 0.30****
Control 637x526 hybrid	5	12.13 ± 0.48	4.07 ± 1.12	1.63 ± 0.25	66.84 ± 2.97	1.97 ± 0.10	12.17 ± 0.49
637x526-Bt-176 hybrid	5	***13.62 ± 0.48****	3.49 ± 1.62	1.68 ± 0.23	68.85 ± 2.29	1.77 ± 0.32	10.24 ± 1.88
Control 684x526 hybrid	5	12.85 ± 0.39	3.66 ± 0.96	1.73 ± 0.16	58.23 ± 7.19	1.56 ± 0.38	12.14 ± 0.28
684x526-Bt-176 hybrid	5	13.32 ± 0.37	***2.04 ± 0.60****	1.63 ± 0.16	***68.07 ± 3.01****	1.61 ± 0.16	***9.01 ± 1.27****
Control 615 inbred	2	10.07 ± 0.15	4.67 ± 0.59	1.73 ± 0.21	63.16 ± 0.93	1.84 ± 0.08	10.82 ± 0.26
615-Bt-176 inbred	2	***11.79 ± 0.07****	4.34 ± 0.13	1.82 ± 0.01	59.14 ± 0.98	1.70 ± 0.22	12.38 ± 0.04
Control 635x615 hybrid	2	11.17 ± 0.62	4.14 ± 0.10	1.93 ± 0.08	61.51 ± 0.75	1.74 ± 0.06	13.22 ± 0.27
635x615-Bt-176 hybrid	2	11.38 ± 0.33	4.05 ± 0.21	1.81 ± 0.01	61.04 ± 1.82	1.92 ± 0.23	12.06 ± 0.10

*: statistically sig difference 5% level pairwise T-test

(ii) Amino acid composition of Bt-176 corn

Sixteen individual amino acids were quantified. The levels of glutamine, asparagine, cysteine and tryptophan were not determined. The data are shown in Tables 10a and 10b. Some small, but statistically significant (5% level in a pairwise T-test), differences were observed for some amino acids. However, the overall amino acid profile was similar for transgenic and isogenic corn. The values for all lines were comparable to typical values reported in the literature (Wright 1987).

Table 10a. Amino acid content of grain from inbred CG00526- 176 Corn % total protein

Component	CG00526 Control	CG00526-176 inbred	Typical literature values[1]
Glutamate	15.74 ± 0.63	15.32 ± 1.06	18.63
Leucine	10.69 ± 0.57	11.08 ± 0.42	11.05
Proline	7.49 ± 0.22	***8.50 ±0.41****	8.84
Alanine	6.29 ± 0.24	6.47 ± 0.24±	8.21
Aspartate	5.67 ± 0.32	5.07 ± 0.47	7.16
Phenylalanine	4.75 ± 1.04	3.87 ± 0.32	4.42
Serine	3.92 ± 0.16	4.03 ± 0.24	4.63
Valine	3.81 ± 0.20	3.70 ± 0.19	4.0
Arginine	3.46 ± 0.28	3.54 ± 0.21	4.42
Glycine	2.96 ± 0.14	***3.30 ±0.16****	3.89
Threonine	2.95 ± 0.15	3.09 ± 0.12	3.26
Tyrosine	2.94 ± 0.46	2.92 ± 0.20	3.47
Isoleucine	2.88 ± 0.33	2.59 ± 0.08	3.58
Lysine	2.34 ± 0.28	2.48 ± 0.29	2.32
Histidine	2.25 ± 0.10	2.23 ± 0.11	2.63
Methionine	1.92 ± 0.10	1.76 ± 0.15	1.58

n= 5, replicate samples from a pooled sample representing multiple plants. ± standard deviation 1: Wright 1987, *: statistically significant difference at 5% level in a pairwise T-test

Table 10b. Amino acid content of hybrid and inbred lines containing the Bt-176 transformation event

Genotype	Glu	Leu	*Pro*	Ala	Asp	Phe	Ser	Val	Arg	Gly	Thr	Tyr	Ile	Lys	His	Met
526	15.74 ±0.63	10.69 ±0.57	7.49 ± 0.22	6.29 ± 0.24	5.67 ± 0.32	4.75 ± 1.04	3.92 ± 0.16	3.81 ± 0.20	3.46 ± 0.28	2.96 ± 0.14	2.95 ± 0.15	2.94 ± 0.46	2.88 ± 0.33	2.34 ± 0.28	2.25 ± 0.10	1.92 ± 0.10
526-Bt 176	15.32 ± 1.06	11.08 ± 0.42	***8.50* ±0.41***	6.47 ± 0.24	5.07 ± 0.47	3.87 ± 0.32	4.03 ± 0.24	3.70 ± 0.19	3.54 ± 0.21	***3.30* ±0.16***	3.09 ± 0.12	2.92 ± 0.20	2.59 ± 0.08	2.48 ± 0.29	2.23 ± 0.11	1.76 ± 0.15
554x526	15.90 ± 0.45	10.82 ± 0.35	7.23 ± 0.23	6.77 ± 0.32	5.60 ± 0.18	4.03 ± 0.18	4.02 ± 0.14	3.66 ± 0.15	3.84 ± 0.20	3.00 ± 0.08	2.84 ± 0.05	2.85 ± 0.17	2.69 ± 0.05	2.36 ± 0.21	2.05 ± 0.05	1.70 ± 0.13
554x526-Bt-176	16.72 ± 0.68	***11.60**** ± 0.48	7.76 ± 0.30	6.82 ± 0.26	5.55 ± 0.25	***4.93* ±0.44***	4.16 ± 0.15	3.87 ± 0.13	3.88 ± 0.18	3.14 ± 0.09	***3.06* ±0.16***	2.84 ± 0.14	***2.93* ±0.13***	2.55 ± 0.16	***2.20* ±0.09***	1.72 ± 0.05
637x526	15.88 ± 1.15	10.66 ± 0.48	7.52 ± 0.51	6.43 ± 0.49	6.07 ± 0.42	5.20 ± 0.42	4.11 ± 0.27	3.83 ± 0.20	3.84 ± 0.26	3.25 ± 0.16	3.20 ± 0.13	3.06 ± 0.17	2.78 ± 0.17	2.99 ± 0.11	2.19 ± 0.15	1.51 ± 0.09
637x526-Bt-176	15.83 ± 0.69	10.84 ± 0.41	7.60 ± 0.46	6.12 ± 0.29	5.52 ± 0.32	4.82 ± 0.44	3.97 ± 0.17	3.50 ± 0.15	***3.09* ±0.19***	3.05 ± 0.19	3.21 ± 0.18	2.87 ± 0.08	2.66 ± 0.19	***2.20* ±0.21***	***1.91* ±0.09***	***1.35* ±0.08***
684x526	16.34 ± 1.15	11.34 ± 0.89	7.96 ± 0.78	6.25 ± 0.35	5.20 ± 0.31	5.48 ± 0.43	4.05 ± 0.28	3.41 ± 0.25	3.03 ± 0.26	2.86 ± 0.13	3.07 ± 0.24	3.23 ± 0.15	2.53 ± 0.34	1.89 ± 0.36	1.88 ± 0.16	1.52 ± 0.09
684x526-Bt-176	16.83 ± 0.88	12.02 ±0.75	7.84 ± 0.42	6.54 ± 0.36	5.02 ± 0.28	5.96 ± 0.18	4.00 ± 0.18	3.27 ± 0.18	2.71 ± 0.22	2.70 ± 0.08	2.92 ± 0.16	3.35 ± 0.18	2.55 ± 0.15	1.45 ± 0.13	1.77 ± 0.08	***1.37* ±0.07***

* statistically sig difference 5% level pairwise T-test

(iii) Fatty acid composition of Bt-176 corn

The proportion of five fatty acids in kernels of hybrid and inbred corn lines containing the Bt-176 transformation event and isogenic controls was determined. The data are shown in Tables 11a and 11b. The relative proportions of the major fatty acids were similar for the transgenic and control lines and there were no statistically significant differences (5% level, pairwise T-test). The levels observed in all lines were within the ranges reported in the literature (Weber 1987).

Table 11a. Comparison of major fatty acids in kernels from control and Bt-176 corn

% of total fatty acid, mean ± standard deviation (range)

Component	CG00526 Control	CG00526-176 inbred	Literature Range[1]
Palmitic 16:0	12.71 ± 0.89 (11.77–14.33)	12.24 ± 0.57 (11.67–12.94)	6-22
Stearic 18:0	2.39 ± 0.58 (1.90–3.34)	2.20 ± 0.21 (1.95–2.48)	1-15
Oleic 18:1	27.09 ± 1.22 (24.45–28.34)	27.90 ± 0.74 (27.03–28.66)	14-64
Linoleic 18:2	55.08 ± 3.45 (50.46–59.47)	55.38 ± 2.35 (52.13–58.08)	19-71
Linolenic 18:3	0.73 ± 0.15 (0.52–0.83)	0.81 ± 0.08 (0.70–0.89)	0.5-2

n=5, replicates from pooled samples of kernels representing multiple plants, 1: Weber 1987, kernels harvested from 1993 field trials in Hawaii.

(iv) Carotenoids

The levels of the carotenoid content, specifically xanthophylls and β-carotene were determined for hybrid and inbred corn lines containing the Bt-176 transformation event and isogenic controls. The data are shown in Tables 12a and 12b. No statistically significant differences (5% level, pairwise T-test) in xanthophylls content were observed between any of the transgenic Bt-176 corn lines. There were no statistically significant differences (5% level, pairwise T-test) for β-carotene levels, except in the original CG00526-176 inbred lines, in which the level of β-carotene was higher than in the CG00526 control line. This difference could be due to differences in the length of storage time of the kernels as carotenoids have been shown to decrease with time in storage (Wright, 1987).

Table 11b. Major fatty acids in kernels of hybrid and inbred lines containing the Bt-176 transformation event *(% total fatty acid, mean ± standard deviation)*

Genotype	n	Palmitic 16:0	Stearic 18:0	Oleic 18:1	*Linoleic 18:2*	*Linolenic 18:3*
Control 526	5	12.71 ± 0.98	2.39 ± 0.58	27.09 ± 1.22	55.08 ± 3.45	0.73 ± 0.15
526-Bt 176	5	12.24 ± 0.57	2.20 ± 0.21	27.90 ± 0.74	55.38 + 2.35	0.81 ± 0.08
Control 554x526	5	14.26 ± 0.61	2.26 ± 0.16	25.76 ± 0.34	54.74 ± 1.98	0.81 ± 0.04
554xBt-176 hybrid	5	13.77 ± 0.98	2.30 ± 0.17	25.69 ± 0.74	55.25 ± 2.73	0.88 ± 0.10
Control 637x526 hybrid	5	12.92 ± 2.11	2.13 ± 0.41	30.04 ± 1.14	50.16 ± 6.92	0.84 ± 0.14
637x526-Bt-176 hybrid	5	12.72 ± 1.14	2.16 ± 0.16	29.40 ± 0.93	51.12 ± 3.65	0.81 ± 0.09
Control 684x526 hybrid	5	13.93 ± 1.65	2.15 ± 0.41	24.54 ± 1.18	55.86 ± 5.41	0.86 ± 0.13
684x526-Bt-176 hybrid	5	14.17 ± 1.69	2.54 ± 0.50	24.39 ± 0.54	54.80 ± 4.64	0.89 ± 0.15

*: statistically sig difference 5% level pairwise T-test

Table 12a. Carotenoid levels in kernels from inbred CG00526- 176 Corn

Component	CG00526 control	CG00526-176 inbred
Xanthophylls	323.6 ± 112.8 (231–512.3)	378.8 ± 37.5 (352.8–416.6)
β-carotene	15.43 ± 1.18 (14.38–17.17)	***17.38 ± 0.33**** (17.03–17.86)

n=5, Mean ± SD (µg/100g sample) (range), *: statistically significant difference in pairwise T-test at 5% level

Table 12b. Carotenoid levels in kernels from hybrid and inbred lines containing the Bt-176 transformation event

Genotype	N	Xanthophylls	*β-carotene*
Control 526 inbred	5	323.6 ± 112.8	15.43 ± 1.18
526-Bt 176 inbred	5	378.8 ± 37.5	***17.38 ± 0.33****
Control 554x526 hybrid	5	377.1 ± 116.6	15.01 ± 0.96
554xBt-176 hybrid	5	371.8 ± 50.0	15.20 ± 2.90
Control 637x526 hybrid	5	284.7 ± 51.4	4.41 ± 4.04
637x526-Bt-176 hybrid	5	180.3 ± 86.2	3.18 ± 1.24
Control 684x526 hybrid	5	237.9 ± 103.0	3.73 ± 0.80
684x526-Bt-176 hybrid	5	152.1 ± 40.8	2.80 ± 0.33
Control 615 inbred	2	3086.2 ± 67.7	60.92 ± 0.13
615-Bt-176 inbred	2	2918.8 ± 314.5	47.88 ± 2.16
Control 635x615 hybrid	2	1808.7 ± 126.3	41.43 ± 1.76
635x615-Bt-176 hybrid	2	1532.4 ± 113.0	29.25 ± 3.75

Mean ± SD (µg/100g sample) (range), *: statistically significant difference in pairwise T-test at 5% level

(v) Conclusions from compositional analyses

Comprehensive data from a range of compositional analyses conducted on kernels from Bt-176 corn and hybrids and the corresponding unmodified, isogenic control lines were presented for assessment. The compositional components measured included proximates (protein, fat, ash, starch, fibre and moisture), amino acid composition, fatty acids profile, and carotenoid levels.

The results of the kernel compositional data do not indicate that there are any substantial differences between corn lines containing the Bt-176 transformation event and the non-transgenic control lines for any of the parameters measured. Some small statistically significant differences were observed in protein, fat, starch, moisture, amino acid content and β-carotene content for some of the Bt-176 corn lines. However these differences were not apparent in all of the transgenic lines containing the novel genes. The values were within ranges previously reported for corn and were not considered to be of either biological relevance for commercially grown corn varieties or of significance in terms of food safety. In further support of the equivalence of the nutritional adequacy of the insect protected Bt-176 corn, additional compositional data has been provided on hybrid lines of different genetic backgrounds which are consistent with data from the original transformant.

Levels of anti-nutrients

The levels of the trypsin and chymotrypsin inhibitors in corn are very low and are not considered nutritionally significant (Wright 1987).

Ability to support typical growth and well-being

Studies evaluated:
Brake JT 1996 Evaluation of transgenic Event-176 *Bt* corn (corn) in broiler chickens. Performing laboratory: North Carolina State University Poultry Education Unit, Raleigh, NC. Sponsor: Ciba Seeds, Agricultural Biotechnology, Ciba-Geigy Corporation, NC, USA.

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. Carefully designed feeding studies in animals may provide further re-assurance 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.

In the case of Bt-176 corn the extent of the compositional and other data provided in the application is considered adequate to establish the nutritional adequacy and safety of the food. The presented data indicates that the composition of Bt-176 corn lines was equivalent to, and would be expected to be equally nutritious as, the non-transgenic control lines. Nonetheless, the applicant also provided an animal feeding study to compare the wholesomeness of Bt-176 and control corn. Although not considered essential for establishing safety in this instance, this animal feeding study has been reviewed as additional supporting data.

An additional study was submitted that supports the safety and nutritional adequacy of food derived from animals fed genetically modified stockfeed.

Chicken feeding study

The data from this study have been published in the scientific literature (Brake and Vlachos 1998).

One thousand two hundred and eighty 1-day-old broiler chicks (Arbor Acres Yieldmaster) were randomly assigned to 32 single sex groups, with 40 birds/group. Transgenic corn (Ciba Seeds' *Bt*- 176 corn-derived hybrid number 5506BTX) and nontransgenic corn (Ciba Seeds' hybrid G4665) were used. Diets were prepared in both pellet and mash form with both the transgenic and nontransgenic corn, with four groups of each sex fed each type of diet (160 birds/sex/diet type). Prior to the study minor compositional differences were noted between the transgenic and nontransgenic corn and the protein content of the two diets was equalized by non-nutritional filler. Diets were formulated with corn as a base to yield standard protein percentages of 22% for starter feed and 20% for grower feed. Growth performance was measured by weight gain, the feed conversion ratio (FCR, feed:gain), and final body weight. Lower FCR values represent more efficient weight gain per unit feed. The results are summarised in Table 13 below.

Table 13. Body weight gain and Feed Conversion Ratio (FCR) in broiler chickens fed Bt-176 corn

Corn Diet	**day 1**	**day 14**	**day 28**	**day 38**
	Mean body weight			
Bt-176	41	375	1213	1825
G4665 control	41	372	1199	1802
FCR				
Bt-176		1.18	*1.51**	1.74
G4665 control		1.19	*1.55**	1.76

* statistically significant difference at 0.05 significance level with a General Linear Model analysis

During the study there were no clinical signs related to treatment with transgenic corn. Survival was very high in all groups (96–98%), with no difference between groups.

There were no significant differences in bodyweight between birds fed the transgenic corn and birds fed the nontransgenic corn at any time period. There was a slightly higher, but statistically significant ($P<0.05$), feed conversion ratio for birds fed on the transgenic corn diet than on the nontransgenic corn at 28 days, but no differences at 14 or 38 days. In birds fed Bt-176 corn there was a statistically significant increase in the amount of *Pectoralis minor* muscle and skin overlying the total breast, further demonstrating the absence of any detrimental effects.

The data demonstrate that the transgenic corn is equivalent to commercial varieties in its ability to support typical growth and well-being in chickens.

Feeding study of Bt-176 and Bt-11 corn in the diet of laying hens

A 14 day study was conducted by Wildlife International Ltd using methods and

species based on procedures specified in the Environmental Protection Agency's Registration Guidelines, *Pesticide Assessment Guidelines, FIFRA Subdivision O, Hazard Evaluation: Pesticide-Residue Chemistry Guidelines*. Single comb, white laying hens (28-week old at start of treatment) were fed diets containing 64% corn meal from Bt-176 or Bt-11 derived genetically modified corn.

The study birds were from the same lot and age and were acclimated to the test facility for 11 days prior to the start of the pre-treatment phase. From this lot, 40 hens were randomly assigned to the control and treatment groups (10 per group).

One group received a diet prepared with Bt-176 grain and a second group received a diet prepared with Bt-11 grain. A third group received a diet prepared with non-genetically modified control hybrid grain (Bt-176) and a fourth group received a diet prepared with non-genetically modified control hybrid grain (Bt-11). The birds were fed the diets *ad libitum* for 14 days and evaluated for survival, body weight and general health. The number and weight of eggs produced were measured daily. Data on feed consumption, egg production and egg weight for each pen was compared to the comparable values from the pre-treatment phase (final 7 days of acclimation). Eggs from the final two days of the study were collected for analysis for the transgenic proteins. Hens from the control and treatment groups were sacrificed at the end of the 14 day exposure period and selected tissues were taken for analysis of the transgenic proteins.

There were no mortalities observed in the control or treatment groups during the course of the study. No effect was observed on survivability, health, egg production or egg weight when compared to birds fed non-modified control corn meal. There were no differences from the pre-treatment phase in the parameters measured. Additionally, the Cry1A(b) and PAT proteins were not detected in any of the five tissue types analysed (egg white, egg yolk, liver, breast and thigh).

When food substances are known to be hazardous, an estimate is made of the dietary intake to determine the likely human exposure to the hazard. If exposure is likely to be low there may be less cause for concern than if exposure is likely to be high. In Bt-11 corn, the dietary exposure estimate has been calculated for the Cry1Ab protein and was not determined for the PAT protein because it was at the limit of detection in corn kernels.

Other information – dietary exposure assessment

Dietary exposure to the Cry1Ab protein from consumption of Bt-176 corn has been estimated. The exposure to the PAT protein was not determined because it was not detected in kernels.

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-176 corn kernels at trace levels that were so low, they could not be quantified, i.e. less than 5 ng protein/g fresh weight kernels. Therefore exposure to the Bt protein from Bt-176 corn is unlikely. However, a case scenario that considers the market penetration of both Bt-11 and Bt-176 corn and using the highest level of the Bt protein found in Bt-11 is discussed below.

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-176/Bt-11 corn and will therefore be an overestimate of the true content of Bt-176/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 protein concentration of the Cry1Ab protein from both corn lines (3.80 µg protein/g fresh weight in Bt-11 corn) 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 Bt corn lines and the other, more realistic but still conservative estimate, where it is assumed that 10% of corn products are derived from Bt-11 and Bt-176 corn. 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.

[2] Calculated for all respondents

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

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-176 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. Bt-176 corn was found to be equally nutritious as conventional corn when used as feed for chickens and no effects on egg weight or production in laying hens. No differences were observed in tissues from laying hens fed genetically modified corn or control corn. Additionally, a dietary exposure assessment demonstrates that exposure to the novel protein from Bt-corn is likely to be small.

Based on the data submitted in the present application, grain derived from Bt-176 corn is nutritionally and compositionally comparable to that from conventional corn and is not considered to pose a risk to human health and safety.

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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

Australia New Zealand Food Authority (ANZFA) 1999a Guidelines for the safety assessment of foods to be included in Standard A18 - food produced using gene technology.

Australia New Zealand Food Authority (ANZFA). *Food Standards Code* 1999b Standard A14 – Maximum Residue Limits.

Astwood, J. 1995 *Bacillus thuringiensis* susp. *kurstaki* HD-1 insecticidal protein (*B.t.k.* HD-1 protein) shares no significant sequence similarity with proteins associated with allergy or Coeliac disease. Monsanto Company, USA 63198. MSL-14172

Benfey, P.N. and Chua, N-H. 1990 The Cauliflower Mosaic Virus 35S promoter: combinatorial regulation of transcription in plants. *Science* 250: 959-966

Beriault, J.N., Horsman, G.P. and Devine, M.D. 1999 Phloem transport of D,L-glufosinate and acetyl-L-glufosinate in glufosinate-resistant and –susceptible *Brassica napus. Plant Physiol* 121: 619-627

Botterman J., V Gossele, C Thoen and M Lauwereys. 1991. Characterisation of phosphinothricin acetyltransferase and C-terminal enzymatically active fusion proteins. Gene 102:33-37

Brake, J. and Vlachos, D. 1998 Evaluation of transgenic event 176 "Bt" corn in broiler chickens. *Poultry Sci* 77: 648-653

Callis, J., Fromm, M. and Walbot, V. 1987 Introns increase gene expression in cultured maize cells. *Genes Dev* 1: 1183-2000

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

Canadian Food Inspection Agency 1995 Novel Food Information – Food Biotechnology: Insect resistant corn, 176, 19 December 1995 Office of Food Biotechnology. http://www.agbios.com/decdocs/ofb-095-353-a.pdf

Canadian Food Inspection Agency 1996 Decision Document DD96-09: Determination of environmental safety of event 176 Bt corn (*Zea mays* L.) developed by Ciba Seeds and Mycogen Corporation. 16 April 1996 Plant Biotechnology Office. http://www.cfia-acia.agr.ca/english/plaveg/pbo/dd9609e.shtml

Cao, J., Duan, X.L., McElroy, D. and Wu, R. 1992 Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Reports* 11: 586-591

Cooper, D. 1991 *Bacillus thuringiensis* toxins and mode of action. *in* The Proceedings from the Workshop on *Bacillus thuringiensis.* Editors R. Milner and C. Chandler. CSIRO, Canberra.
Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D.H. 1998 Revision of nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62: 807-813

De Block, M., Bottermna, J., Vanderwiele, M., Dockx, J., Thoen, C., Gossele, V., Rao Movva, N., Thompson, C., Van Montagu, M., and Leemans, J. 1987 Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J* 6: 2513-2518

Drummond, J. and D. Pinnock. 1991. Host spectrum of *Bacillus thuringiensis.* In *The Proceedings from the Workshop on* Bacillus thuringiensis*.* Editors R. Milner and C. Chandler. CSIRO, Canberra.

Ebert, E., Leist, K.H. and Mayer, D. 1990 Summary of safety evaluation toxicity studies on glufosinate ammonium. *Food Chem Toxicol* 28: 339-349

Estruch, J.J., Kadwell, S., Merlin, E. and Crossland, L. 1994 Cloning and characterization of a maize pollen-specific calcium dependent calmodulin-independent protein kinase. *Proc Natl Acad Sci USA* 91: 8837-8841

Fearing, P.L., Brown, D., Vlachos, D., Meghji, M. and Privalle, L. 1997 Quantitative analysis of Cry1A(b) expression in *Bt* maize plants, tissues, and silage and stability of expression over successive generations. *Mol Breed* 3: 169-176

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.

Gallo-Meagher, M. and Irvine, J.E. 1996 Herbicide resistant sugarcane plants containing the *bar* gene. *Crop Sci* 36: 1367-1374

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

Gill, SS. 1995 Mechanism of action of *Bacillus thuringiensis* toxins. *Mem Inst Oswaldo Cruz* 90:69-74

Hack, R., Ebert, E., Ehling, G. and Leist, K.H. 1994 Glufosinate ammonium – some aspects of its mode of action in mammals. *Food Chem Toxicol* 32: 461-470

Hadley, W.M., Burchiel, S.W., McDowell, T.D., Thilsted, J.P., Hibbs, C.M., Whorton, J.A., Day, P.W., Friedman, M.B. and Stoll, R.E. 1987 Five-month oral (diet) toxicity/infectivity study of *Bacillus thuringiensis* insecticides in sheep. *Fundam Appl Toxicol* 8: 236-242

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

Hudspeth, R.L. and Grula, W. 1989 Structure and expression of the maize gene encoding the phosphoenolpyruvate carboxylase isozyme involved in C_4 photosynthesis. *Plant Mol Biol* 12: 579-589

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

Klein, R.M., Wolf, E.D., Wu, R. and Sandford, J.C. 1992 High-velocity microprojectiles for delivering nucleic acids into living cells. *Biotechology* 24: 384-386

Komari, T., Hiei, Y., Ishida, Y., Kumashiro, T. and Kubo, T. 1998 Advances in cereal gene transfer. *Curr Opin Plant Biol* 1: 161-165

Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M.R., Merlin, E., Rhodes, R., Warren, G.W., Wright, M. and Evola, S.V. 1993 Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio/Technology* 11: 194-200

Koziel, M.G., Carozzi, N.B. and Desai, N. 1996 Optimizing expression of transgenes with an emphasis on post-transcriptional events. *Plant Mol Biol* 32: 393-405

Kues U and U Stahl. 1989 Replication of plasmids in gram-negative bacteria. *Microbiol Rev* 53:491-516

Kumada, Y., Anzai, H., Takano, E., Murakami, T., Hara, O., Itoh, R., Imai, S., Satoh, A. and Nagaoka, K. 1988 The bialaphos resistance gene (*bar*) plays a role in both self-defense and bialaphos biosynthesis in *Streptomyces hygroscopicus*. *J Antibiot (Tokyo)* 41: 1838-1835

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

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

Merriman, T.N. 1996 An Acute Oral Toxicity Study in Mice with Phosphinothricin Acetyltransferase (PAT) Protein. DEKALB Genetics Corporation, 62 Maritime Drive, Mystic, CT 06355-1958. DEKALB Study No. DGC-95-A18.

Neu, H.C. 1992 The crisis in antibiotic resistance. *Science* 257:1064-1073

Noteborn H.P., Bienenmann-Ploum M.E., van den Berg J.H., Alink G.M., Zolla L., Reynaerts A., Pensa M. and Kuiper H.A. 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, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L., Fischhoff, D.A. 1991 Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc Natl Acad Sci USA* 88: 3324-3328

Rajamohan, F., Lee, M.K., and Dean, D.H. (1998) *Bacillus thuringiensis* insecticidal proteins: molecular mode of action. *Prog Nucleic Acid Res Mol Biol* 60: 1-27

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

Sanders, P.R., Lee, T.C., Groth, M.E., Astwood, J.D. and Fuchs, R.L. 1998 Safety assessment of insect-protected corn. *in* Thomas, J.A. (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

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

United States Department of Agriculture, Animal and Plant Health Inspection Service. 1995 USDA/APHIS Petition 94-319-01 for determination of nonregulated status for Event 176 corn. Environmental assessment and finding of no significant impact. http://www.agbios.com/decdocs/9431901p.htm

United States Environmental Protection Agency 1997 Phosphinothricin Acetyltransferase and the genetic material necessary for its production in all plants; exemption from the requirement of a tolerance on all raw agricultural commodities. Federal Register Volume 62 Number 70 pp17717-17720. http://www.epa.gov/fedrgstr/EPA-PEST/1997/April/Day-11/p9373.htm

United States Environmental Protection Agency 1982. *Pesticide Assessment Guidelines, FIFRA Subdivision O, Hazard Evaluation: Pesticide-Residue Chemistry Guidelines,* subsection 171-4, Environmental Protection Agency, Office of Pesticide Programs. Washington , D.C.

United States Food and Drug Administration 1999 Foods derived from new plant varieties derived through recombinant DNA technology: final consultations under FDA's 1992 policy. Office of Premarket Approval, Center for Food Safety & Applied Nutrition, US FDA http://vm.cgscan.fda.gov/~lrd/biocon.html

Watanabe, T. and Sano, T. 1998 Neurological effects of glufosinate poisoning with a brief review. *Hum Exp Toxicol* 17: 35-39

Watson S.A. 1987. Structure and composition. *in* Corn: Chemistry and Technology. ed. Watson, S.A. and Ramstead, P.E. American Association of Cereal Chemists Inc, St Paul, Minnesota.

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

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., Broer, I., Hillemann, D., Strauch, E. and Puhler, A. 1988 Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Strepromyces viridochromogenes* Tu494 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