



Estudio de caso 1

Evaluación de la inocuidad del maíz genéticamente modificado resistente a los insectos, evento MON 810

Food safety assessment of genetically modified insect resistant corn event MON 810

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Preface

The United States Food and Drug Administration (FDA) completed a consultation for insect resistant (protected) corn line MON 810 in 1996. Health Canada notified Monsanto that the Department had no objection to the food use of corn line MON 810 in 1997. These decisions were made by both regulatory authorities following a comprehensive assessment of MON 810 based upon internationally accepted principles for establishing the safety of foods derived from genetically modified plants. The record of review and decision-making is described for the FDA consultation in Appendix 1 and for Health Canada's assessment in Appendix 2.

The data and information in this case study have been summarized for training purposes. The case study is derived from parts of the food safety submission assessed by Health Canada. Monsanto Canada Inc. provided data on the description of the new variety, the donor organism(s), the genetic modification methods and characterization. The novel protein was identified, characterized and compared to the original bacterial protein, including an evaluation of its potential toxicity. Scientific publications and data from field testing in Canada and the United States under confined trials in 1995 and 1996 were supplied.

Note that statements in quotes are taken directly from the submission to Health Canada.

Disclaimer

Monsanto Canada Inc. has consented to the use of the information provided in their regulatory submission for event MON 810 as a training tool. It must be noted, however, that in order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original applications. Certain information has been reduced to summaries and the present data as presented in the case study are only a subset of that actually submitted. The case study in no way constitutes a complete application nor is it to be considered a complete safety assessment. To that end, the use of this information in the form of a training tool does not constitute an endorsement of the information or product nor should it be considered a reflection of any of the original submissions.

Description of the recombinant-DNA plant

Line MON 810 contains an inserted genetic fragment of the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 that produces an active delta endotoxin protein expressed in the corn tissue. The target pest, European corn borer (ECB) (*Ostrinia nubilalis*), is an important corn insect pest. Physical damage is caused by ECB feeding on various tissues of the corn plant. The tissues damaged depend on the number of generations of ECB. The damage from ECB feeding includes: a) leaf feeding, b) stalk tunneling, c) leaf sheath and collar feeding, and d) ear damage. Estimated losses range from 5-10% corn yield annually from ECB from disruption of nutrient and water translocation, secondary disease infections, stalk lodging, ear droppage and kernel damage.

The company further describes the variety and its history, "Line MON 810 was supplied to various seed companies as F1 seed of transformed genotype Hi-II crossed to several various elite inbreds. The resulting lines were subjected to multiple cycles of backcrossing to the recurrent inbred parent to recover the converted elite genotype, followed by several cycles of selfing to derive converted inbred parents for hybrid testing. Further cycles of seed increase (selfing) are required to produce parent seed for commercial hybrid seed production. Insect-protected hybrid seed will be heterozygous for the *cryIA(b)* gene since one inbred parent containing the gene is sufficient to confer the insect-protected phenotype on progeny hybrids."

MON 810 is a field corn, not a sweet corn and is intended primarily as an animal feed, but some human food uses occur for field corn. For example, MON 810 may be used either dry or wet milled in processed corn products for humans. No differences in the intended uses of MON 810 are expected as compared to existing field corn hybrids.

Description of the host plant and its use as food

The host plant used is a hybrid line of *Zea mays* with a Mo17X (Hill X B73) background. These corn lines have a long history of use in particular as animal feed, being field corn and not sweet corn.

Zea mays L. (corn, maize) has been cultivated for over 8000 years in Mexico and Central America. A versatile and responsive species, corn has increased both in productivity and geographical range over the past century

with the development of hybrids, breeding programs and fertilizer use and is now grown on every habitable continent. Corn yields prior to hybridization in the early 1930s were around 1.3 metric ton per hectare (ha). The current record high is 123.5 t/ha (with an average of around 137 bushels per acre in the US). World production of corn in 2000 is estimated at 23,800 million bushels.

Corn is used for many different products and uses, as a staple food in many parts of the world and in derived forms, such as starch, alcohol, oil, and for animal feed. Also, corn is used for production of ethanol as a renewable fuel.

Description of the donor organism(s)

The donor of the *cryIA(b)* gene that codes for the CryIA(b) protein, a delta endotoxin active against lepidopteran insect pests, is *Bacillus thuringiensis* subsp. *kurstaki* (B.t.k.) strain HD-1.

The *cryIA(b)* gene inserted into MON 810 originates from a *Bacillus thuringiensis* subsp. *kurstaki*. *Bacillus thuringiensis* (or Bt) species are spore-forming, gram-positive bacteria that produce a crystal with insecticidal properties. Bt species have been used commercially as pest control agents for decades.

Different strains of Bt are insecticidally active against selected insect pests:

- Bt *israelensis* strains for dipterans (mosquitoes and black flies)
- Bt var. *sandiego* and *tenebrionis* strains for coleopterans (Colorado potato beetle, elm leaf beetle, yellow mealworm)
- Bt *kurstaki*, *thuringiensis*, *sotto* and *aizawai* strains for lepidopterans (corn borer, tomato hornworms, gypsy moth, cabbage looper, tobacco budworm, cotton bollworm).

The delta endotoxin crystals are produced when the bacterium sporulates. To be active, the protein must be ingested by the insect. While the protein is insoluble at neutral or acidic pH, it is soluble at the alkaline pH that occurs in the guts of larval insects where it is activated by proteases in the gut. The activated protein (stripped of its carboxy terminal and about 28 amino acids from the amino terminal end, at approximately 600 amino acids in size) diffuses through the peritrophic membrane of the insect to the midgut epithelium. There it binds to the specific high affinity receptors on the surface of the insect midgut, inserts itself into the membrane and forms ion-specific pores (non-target insects, birds, mammals and fish do not have these

receptors). The resulting pores in the membrane cause leakage of the intracellular contents into the gut lumen and water into the epithelial gut cells which swell and lyse. The gut becomes paralyzed disrupting the digestive process, which causes the insect to stop eating and die.

The protein produced in MON 810 insect protected (IP) corn is identical to that produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, which controls insect pests by the production of delta-endotoxin crystals. Data to support this claim are supplied in the submission.

B.t.k. has been used as a microbial pest control agent for decades and “the naturally occurring Bt proteins have been demonstrated to be virtually non-toxic to fish, avian species, mammals and other nontargets ... no adverse effects are expected to wildlife from the commercialization of these plants.”

The company’s submission states: “The CryIA(b) protein is insecticidal only to lepidopteran insects. Only seven of the eighteen insects screened were sensitive ... and they were all lepidopteran. This specificity is directly attributable to the presence of receptors in the target insects. Selective activity of B.t.k. endotoxin will not disrupt populations of either beneficial insects or nontarget animals (e.g., birds, fish).”

Tests (cited from the literature), registration documentation and safety assessments from pesticidal registrations on commercially available microbial pesticide products, such as DIPEL®, indicate that they are “widely recognized as nontoxic for mammals, birds and fish as well as beneficial nontarget insects including predators and parasitoids of lepidopteran insect pests and honeybee.”

Description of the genetic modification

Plasmid DNA was introduced into the plant tissue by particle acceleration (also known as biolistic transformation). The DNA is precipitated onto the surface of microscopic tungsten or gold particles using calcium chloride and spermidine. A drop of coated particles, placed onto a plastic macrocarrier, is accelerated at high velocity through a barrel by a gunpowder explosion. The macrocarrier flight is stopped by a plastic stopping plate allowing the DNA-coated particles to continue their journey, penetrating plant cells in the path of the explosion. The DNA is deposited and incorporates into the cell chromosome. The cells are incubated on a tissue culture medium containing 2,4-D, which supports callus growth. The cells with introduced DNA contain genes for glyphosate tolerance and are

grown in the presence of glyphosate to select the transformed cells.

Two plasmids were used during this biolistic process, PV-ZMBK07 (Figure 1) containing the *cryIA(b)* gene and PV-ZMGT10 (Figure 2) containing two marker genes used for selection on glyphosate, CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) and glyphosate oxidoreductase (*gox*). Tables 1 and 2 describe the DNA elements in the plasmids.

Only a portion of the PV-ZMBK07 plasmid vector is present in MON 810 and the final MON 810 construct does not contain the marker genes. Details on how this was determined follow in Chapter 3. “It is presumed that the genes which allow for selection on glyphosate were

originally incorporated into the plant genomic DNA but were lost by segregation during backcrossing.” The reason given is that these genes “integrated at a separate loci from the *cryIA(b)* gene and segregated out during the crossing.”

While both plasmids contain the *nptII* gene encoding for neomycin phosphotransferase II (*nptII*) under the control of its own bacterial promoter, data shows that the *nptII* gene is not present in MON 810. This bacterial gene was used as a selectable marker during plasmid construction.

Experiments in corn transformation have demonstrated that the frequency of obtaining transformants containing glyphosate tolerance selection

Table 1. Summary of DNA elements in plasmid PV-ZMBK07 (See Fig. 1)

Genetic element	Size Kb	Function
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
<i>hsp 70</i> intron	0.80	Intron from the maize <i>hsp70</i> gene (heat shock protein) present to increase the level of gene transcription
<i>cryIA(b)</i>	3.46	The gene encodes the CryIA(b) protein product
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
<i>lacZ</i>	0.24	A partial <i>E. coli lacI</i> coding sequence, the promoter <i>P_{lac}</i> and a partial coding sequence for β-D-galactosidase or <i>lacZ</i> protein from pUC119
<i>ori-pUC</i>	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in <i>E. coli</i>
<i>nptII</i>	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid

Table 2. Summary of DNA elements in plasmid PV-ZMGT10 (See Fig. 2)

Genetic element	Size Kb	Function
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
<i>hsp 70</i> intron	0.80	Intron from the maize <i>hsp70</i> gene (heat shock protein) present to increase the level of gene transcription
CTP2	0.31	Chloroplast transit peptide (CTP) isolated from <i>Arabidopsis thaliana</i> EPSPS present to direct the CP4 EPSPS protein to the chloroplast, the site of the aromatic amino acid synthesis
CP4 EPSPS	1.4	The gene for CP4 EPSPS, isolated from <i>Agrobacterium</i> sp strain CP4 which allows for the selection of transformed cells on glyphosate
CTP1	0.26	Chloroplast transit peptide (CTP) isolated from the small subunit gene of ribulose-1,5-biphosphate carboxylase (SSU1A) gene from <i>Arabidopsis thaliana</i> present to direct the GOX protein to the chloroplast, the site of the aromatic amino acid synthesis
<i>gox</i>	1.3	The gene encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase (GOX) isolated from <i>Achromobacter</i> sp. (new genus <i>Ochrobactrum anthropi</i>) strain LBAA
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
<i>lacZ</i>	0.24	A partial <i>E. coli lacI</i> coding sequence, the promoter <i>P_{lac}</i> and a partial coding sequence for β-D-galactosidase or <i>lacZ</i> protein from pUC119
<i>ori-pUC</i>	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in <i>E. coli</i>
<i>nptII</i>	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid

was increased when both plant selectable markers were used.

The plasmid size of PV-ZMBK07 is 7794 bp and of PV-ZMGT10 is 9427 bp.

Characterization of the genetic modification

Introduction

Several methods, including Southern and Western blot analyses, were used in the molecular characterization of MON 810. Possible novel genes and potential gene products that may have been present in MON 810, based on the information in the plasmid maps, are listed in Table 3.

Molecular characterization

Molecular characterization of the integrated DNA (I-DNA) included determination of:

- The insert number (number of integration sites within the corn genome)
- Copy number (number of each gene within the integrated DNA)
- Insert integrity.

Southern blot analysis was used to determine the above parameters.

MON 810 is compared against a non-transgenic control (counterpart) MON 818, which also has a Mo17 X (Hi-II X B73) background. MON 818 does not contain the genes encoding for B.t.k. HD-1 Cry1A(b), CP4 EPSPS or GOX proteins.

Table 3. Possible novel genes and potential gene products in MON 810.

<i>Novel gene</i>	<i>Novel gene product</i>	<i>Regulatory sequence</i>	<i>Other DNA sequences</i>
PV-ZMBK07			
<i>cryIA(b)</i>	<i>Bt</i> gene	Sequence is controlled by E35S promoter (0.6Kb) and a 0.8 Kb intron from the hsp70 gene (heat shock protein) is present to increase the levels of gene transcription. A 0.24 Kb nopaline synthase 3' nontranslated terminator sequence (NOS 3') attached to the <i>cry</i> gene provides the mRNA polyadenylation signals.	
<i>lacZ-alpha</i>	Betagalactosidase. A polylinker (region with multiple cloning sites) which allowed the cloning of the desired genes in the plasmid vector	Bacteria controlled promoter. Joined at the 3'end of NOS.	Followed by a 0.7 Kb region of replication for the pUC plasmids (<i>oriPUC</i>) which allows replication of plasmids in <i>E. coli</i> .
<i>nptII</i> (marker for selection during construction of the plasmid derived from procaryotic transposon Tn5)	Neomycin phosphotransferase Resistance to aminoglycoside antibiotics (<i>i.e.</i> , kanamycin and neomycin)	Has its own bacterial promoter	
PV-ZMGT10			
<i>gox</i> gene cloned from <i>Achromobacter</i> sp. strain LBAA	Glyphosate metabolizing enzyme, glyphosate oxidoreductase (GOX). Degrades glyphosate by conversion to aminomethylphosphonic acid and glyoxylate	Joined to CTP1 peptide which targets the gene to the plastids, a chloroplast transit peptide. Derived from a subunit of ribulose - 1,5 biphosphate carboxylase (SSU1A) gene from <i>Arabidopsis thaliana</i> . Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	
CP4 EPSPS Isolated from <i>Agrobacterium</i> species strain CP4 which is resistant to glyphosate	5-enolpyruvylshikimate-3-phosphate synthase	Joined to CTP2 peptide. Isolated from <i>Arabidopsis thaliana</i> EPSPS. The gene and CTP2 are about 1.7Kb in size. Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	
Also contains the same <i>lacZ-alpha</i> , <i>ori-pUC</i> and <i>nptII</i> genes described above			

Insert Number

After digestion of extracted DNA with restriction enzyme NdeI, which does not cleave within either of the plasmids used to produce MON 810, analysis shows that a single band at approximately 5.5 Kilobase (Kb) was observed (Figure 3). This indicates that the DNA from the plasmid was present at one site. The rationale for this is that since there are no restriction sites inside the plasmids, the enzyme cleaves outside the inserted DNA releasing a fragment containing the inserted DNA and some adjacent genomic DNA. Since the plasmid DNA inserts randomly in the DNA of the plant, the distance between the inserted DNA and the restriction enzyme sites in the plant DNA will vary. If there are multiple insertion sites it is likely that cutting with a restriction enzyme that cleaves only outside the insert, the released fragment containing the inserted DNA would vary in size depending on the distance from the NdeI restriction site. You would expect to see multiple bands detected in the Southern if there were multiple insertion sites.

Insert Composition

Using a number of probes, tests show that the CP4 EPSPS, *gox* and ori-pUC sequences were not detected in MON 810, whereas *nptII*, E35S, *hsp70* and the *cryIA(b)* were present within the 5.5 Kb NdeI fragment.

cryIA(b)

Digestion of DNA with NcoI/EcoRI to release the *cryIA(b)* gene followed by Southern blot analysis found an approximately 3.1 Kb fragment (Figure 4), which is “sufficient to encode an insecticidally active *CryIA(b)* protein.” While “the positive hybridization control (lane 1 of figure 4) produced one 3.46 Kb fragment which corresponds to the expected size of *cryIA(b)* gene, the MON 818 DNA (lane 2) does not contain any bands, as expected for the control line. The MON 810 DNA contains one band of approximately 3.1 Kb.”

Western blots indicate that the trypsin resistant protein of 63 Kilo-Dalton (kD) is produced by the integrated partial *cryIA(b)* gene in MON 810 (Figures 5 and 6). “Based on the Western blot data and efficacy of maize line MON 810, the *cryIA(b)* gene present produces an insecticidal *CryIA(b)* protein which provides effective, season long control of ECB.”

CP4 EPSPS

Digestion with NcoI/BamHI would release any CP4 EPSPS genes present. Southern blots (Figure 7) indicate

that MON 810 does not contain the 3.1 Kb fragment (the expected size of CP4 EPSPS) found in the gel spiked with the two plasmids. The CP4 EPSPS protein was not detected by ELISA in leaf, whole plant or grain tissues. Western blot analysis confirms the absence of the protein from leaf extracts (Figure 8, lane 9).

gox

Digestion with NcoI/BamHI would excise the *gox* gene, if present (NcoI to NcoI) and would be about 3.1 Kb in size. Southern blot analysis (Figure 7) indicates that MON 810 does not contain the *gox* gene. Neither was it detected by ELISA of plant tissues nor by Western blot analysis (Figure 9, lane 8).

Plasmid backbone

In order to detect backbone (*nptII*/ori-pUC) DNA, the *nptII* gene was used to probe a NcoI/EcoRI digestion of the MON 810 DNA and PV-ZMBK07 plasmid DNA. When probed with the *nptII* gene, Southern analysis detected bands only for the plasmid at 2.5 Kb and 1.8 Kb. No signal was detected in the MON 810 DNA. Using the ori-pUC DNA a 1.8 Kb band for detected in the plasmid lane, but the ori-pUC Southern blots (Figure 10) indicate that MON 810 contains no ori-pUC backbone sequences.

From the above information the interpretation is that one I-DNA containing approximately 4 Kb of DNA from the PV-ZMBK07 plasmid consisting of a portion of the enhanced E35S promoter (estimated to include one of two enhancer elements plus the promoter), the full length intron from the *hsp70* gene (heat shock protein) and 2448 bp of the full length of 3468 bp *cryIA(b)* gene was inserted in the genome of MON 810, as shown in the schematic in Figure 11. No DNA from the bacterial vector backbone (*e.g.*, the pUC-origin of replication), the *nptII*, *gox* or CP4 EPSPS genes was detected. The submission states that, “MON 810 contains one integrated DNA contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, maize *hsp70* intron and the *cryIA(b)* gene.” Western analysis established that the trypsin resistant 63 kD B.t.k. HD-1 protein was produced in MON 810.

CryIA(b) gene integrity and activity

During particle acceleration plasmid DNA can be broken, resulting in integration of partial genes into the genomic DNA. Southern blots and genomic clone sequence established that the first 2448 bp of the 3468 bp *cryIA(b)* gene integrated into MON 810.

Modified plant expression

Molecular analysis of MON 810 “established that the line only contains *cryIA(b)* gene from plasmid PV-ZMBK07 and not the CP4 EPSPS, *gox* or *nptII/ori-pUC* genes. There is no evidence that any of the DNA contained in plasmid PV-ZMGT10 was inserted. MON 810 contains one integrated DNA fragment, contained on a 5.5 Kb *NdeI* fragment, which contains the E35S promoter, the maize *hsp70* intron and the *cryIA(b)* gene.”

The ‘*cry1a(b)*’ gene and its novel trait

The full length gene encoding for CryIA(b) protein has been described. While the genes inserted into MON 810 have been modified to enhance expression in corn, the amino acid sequence of expressed protein is identical to natural protein derived from B.t.k. The *cryIA(b)* gene fragment (Table 4) inserted into the MON 810 has been shown to be equivalent to the original bacterium source, as far as activity against insect pests. Table 4 is a summary of the gene product and its characteristics as submitted by the company.

Western analysis was used:

- To assess the protein products of the partial gene using antibodies specific to B.t.k. proteins
- To compare them to the *E. coli* produced protein standard and tissue extracts from other insect protected corn lines
- To look for any anomalous or unexpected protein products (ex. CP4 EPSPS and GOX (Figures 8, 9, and 12)), and
- To determine if the expressed B.t.k. protein was converted to the expected size of 63 kD trypsin-resistant protein product (Figures 5 and 6).

The company stated, “as is commonly observed in Western blot analysis of Bt proteins, multiple protein products were observed for line MON 810 and the other six insect protected corn lines (Figure 5, lanes 5-11). The full-length gene was not observed in line MON 810, as expected since the full-length gene was not incorporated into the corn genome. ... MON 810 showed no apparent

differences in the size ranges of the less than full length protein products ... when compared to the other six insect protected lines produced with the same full length *cryIA(b)* gene. The predicted molecular weight of the B.t.k. HD-1 protein from the partial *cryIA(b)* gene is 92 kD but is not detected, probably due to low expression or rapid degradation to the trypsin-resistant product during the extraction process.”

When the protein extracts are subjected to trypsin digestion, all seven lines show the core protein at approximately 63 kD (Figure 6).

The protein products in MON 810 and expected immuno-reactive products are similar to those in other IP corn lines, except for the lack of the full length B.t.k. HD-1 protein. No unexpected products were observed. The trypsin results demonstrate that the partial *cryIA(b)* gene inserted into MON810 produces the efficacious trypsin-resistant B.t.k. HD-1 protein.

Equivalence of bacterial and plant produced protein

Escherichia coli containing the B.t.k. gene was used to produce the quantities of the CryIA(b) protein needed to do tests, such as feeding trials. Therefore, the equivalence of the B.t.k. HD-1 protein produced in the IP corn was assessed against that from the *E. coli*. As the company states, the rationale is that: “the expression level of B.t.k. HD-1 in IP corn plants is extremely low. Therefore it is not feasible to isolate this protein from plants in sufficient quantity to conduct the various safety studies performed for the registration of this product. The best alternative was to isolate the functionally active B.t.k. HD-1 protein produced in a microbial host ... and verify its physical and functional equivalence to the plant-expressed protein. Because the full length B.t.k. HD-1 protein (~ 131 kD) ... would be expected to be rapidly converted to the trypsin-resistant core protein (~ 63 kD) upon ingestion ... the trypsin-resistant core of the B.t.k. HD-1 protein was considered an appropriate test material to assess the full length B.t.k. HD-1 protein.”

Two studies were presented. One study compares the B.t.k. HD-1 CryIA(b) from the commercial microbial

Table 4. Summary of gene products in the modified plant

Gene product	Breakdown products, byproducts and metabolic pathways	Expression	Activity of the gene product in the plant	Activity of the gene product in the environment
CryIA(b) delta endotoxin protein	Tryptic peptide is active ingredient	Constitutive	Does not affect other metabolic pathways	Rapidly degraded by digestion (non lepidopteran) and in soil

product DIPEL with leaf tissue samples from the plant expressed in line 754-10-1. Line 754-10-1 was produced with the same transformation plasmids as MON 810, but has higher expression of the protein and therefore it was possible to purify a greater quantity of the protein for equivalence studies. The study demonstrated that the B.t.k. HD-1 trypsin resistant core from corn and *E. coli* are equivalent in molecular weight and immunological reactivity. Both DIPEL and line 754-10-1 contain a full length B.t.k. protein band at approximately 134 kD and the same trypsin resistant core of approximately 63 kD. Western blots demonstrated that the B.t.k. HD-1 core from line 754-10-1 and MON 810 were equivalent, therefore it is concluded that the protein produced by the *E. coli* is an appropriate substitute for the protein in MON 810.

Multiple protein products occur in the plant extract, in the commercial microbial product DIPEL and in the full-length protein preparation used in the acute toxicity study. A question about other fragments in the Western blots that are reactive to the CryIA(b) antibody probes and the meaning were addressed with the following. There should be no concerns since the acute oral toxicity study would have included these fragments. Any fragments outside the trypsin resistant core 28-610 amino acids (1-28 and 611-1150) possibly present in corn tissues show no amino acid homology with known toxins or allergens. Comparison of the CryIA(b) full length protein sequence against the same sequence data base indicates there is no homology with known toxins or allergens. Digestive fate shows that the protein is rapidly digested and the commercial microbial product DIPEL contains many fragments as well.

Western blots of proteins after treatment with trypsin show equivalent bands and that the 63 kD core is in both samples. MON 810 produces a protein product whose trypsin resistant core is equivalent to the trypsin resistant core of the B.t.k. 754-10-1 protein in terms of size and activity.

In a newer test than the one for 754-10-1, the equivalency was established directly between the bacterially and plant produced proteins in MON 810 using Western blot analysis, which was, “highly sensitive, specific for B.t.k. proteins and allows for comparison of the apparent molecular weights of proteins possessing immunological cross-reactivity in complex mixtures.”

Leaf extracts of several IP lines and control lines were digested in trypsin to produce their B.t.k. HD-1 trypsin-resistant core protein and compared against the 63 kD *E. coli* produced trypsin-resistant core protein

and the reference corn line MON 801 protein. The corn lines included MON 810 and its counterpart MON 818.

The Western blot analysis (Figure 6) shows a prominent band at the same molecular weight for MON 810 as the bacterial reference material. Smaller bands are also present and are assumed to be other B.t.k. HD-1 fragments. A band at 20 kD was seen in all extracts (both IP and control lines) and presumably represents a background non-specific cross-reactivity unrelated to the B.t.k. HD-1 protein.

“The results obtained in this study clearly establish that the B.t.k. HD-1 protein (as the trypsin-resistant core) produced by both *E. coli* and the IP corn lines analyzed in this study are equivalent. ... the equivalence established ... serves as the justification for using the safety data generated with the *E. coli*-produced (lot #I92017) protein to support the safety of the B.t.k. HD-1 protein expressed in these new insect protected corn lines.”

Expression

Samples of field-grown IP corn (MON 810) and a control (MON 818) collected from US field sites were used to assess the expression level of CryIA(b), CP4 EPSPS, GOX and NPTII proteins. The control lines (MON818 and 819) are not genetically modified, but have “background genetics representative of the test substances.” MON 818 is the counterpart for MON 810.

Leaf and grain samples were collected from six field sites distributed across the US corn growing regions, representative of the conditions where IP corn could be grown as a commercial product (2 in Illinois, 2 in Iowa, 1 each in Indiana and Nebraska). Whole plant and pollen samples were collected once from a single site (in Illinois). Over season leaf samples (taken every two weeks) were also collected from the Illinois site. Except for the pollen samples, B.t.k. HD-1, CP4 EPSPS and GOX protein levels were assessed using validated ELISAs specific for each protein. For the pollen samples, ELISA was used for the B.t.k. levels and Western blot analysis for CP4 EPSPS and GOX proteins.

Expression levels of the *cryIA(b)* gene were low in corn leaf, seed, pollen and whole plant tissues (Table 5). CP4 EPSPS, GOX and NPTII proteins were not detected. Average protein expression evaluated at six locations was 9.35 µg/g (f.w.) in leaves and 0.31 µg/g (f.w.) in seeds. Protein expression evaluated at one site was 4.15 µg/g (f.w.) in the whole plant and 0.09 µg/g (f.w.) in pollen, as determined from a single sample. Protein expression ranged from 7.93 to 10.34 µg/g (f.w.) in leaves, from 0.19 to 0.39 µg/g (f.w.) in grain and from

Table 5. Summary of levels of protein expression in MON 810 tissues¹

<i>Tissue</i>	<i>Mean</i>	<i>Standard deviation</i>	<i>Range</i>
B.t.k. HD-1			
Leaf	9.35	1.03	7.93-10.34
Over season leaf ²	9.78, 8.43, 4.91		
Pollen	0.09		
Whole plant ³	4.15	0.71	3.65-4.65
Grain	0.31	0.09	0.19-0.39
CP4 EPSPS			
Leaf, over season leaf ² , whole plant, grain	nd	–	–
GOX			
Leaf, over season leaf ² , whole plant, grain	nd	–	–

¹ Unless indicated, values are in µg/g fwt (fresh weight). Unless indicated, the mean, standard deviation and range were over the six sites sampled. For those samples collected at one site see other notes.

² The numbers are means for the three separate sampling times collected at two week intervals.

³ The mean and standard deviation were calculated from one site.

3.65 to 4.65 µg/g (f.w.) in the whole plant. Protein expression declined over the growing season as indicated by the CryIA(b) levels present in leaves assayed over the growing season.

Tissue specificity, as stated by the company, was not expected since the *cryIA(b)* gene is “under the control of a CaMV promoter. Since this is a constitutive promoter that is not developmentally or tissue restricted, no specificity of expression to particular tissues is anticipated, although the CaMV promoter may be more or less active in certain cell types, as seen from the distribution of the CryIA(b) proteins in tissues.” Neither were developmental stage specificity nor inducibility expected or found, because the CaMV promoter is a non-inducible constitutive promoter.

Western blot analysis of pollen (Figure 12) shows that the GOX gene is not expressed in MON 810 (lane 11).

For GM food assessments, expression in the consumed portion of the plant, in this case the grain, is the most important. The levels of expression in the grain of the novel protein range from 0.19 to 0.39 µg/g fresh weight.

The expression of the NPTII protein from the *nptII* gene, under the control of a bacterial specific promoter was tested for one of the lines used in this test (MON 801). The promoter was not active and, therefore, the gene does not express the protein in plant cells.

Breakdown products and metabolism

“The CryIA(b) protein does not have any specific breakdown products in plants. In the insect gut, the alkaline environment solubilizes the protein, which is

then cleaved by proteases to yield the activated endotoxin. ... As is commonly observed in Western blot analysis of Bt proteins, multiple polypeptides are apparent in extracts of plants expressing the *cryIA(b)* gene. These are recognized as breakdown products liberated as a result of protease action either in planta or during extraction.”

Stability of the insert

MON 810 has been crossed into diverse corn genotypes for several generations and the efficacy of the line has been maintained. The molecular characterization of MON 810 was from the third generation of backcrossing and therefore the single insert appears to be stably integrated. Segregation data (Table 6) support a single active insert of the *cryIA(b)* gene segregating according to Mendelian genetics.

The *cryIA(b)* gene is stable through seven generations of crosses to one recurrent parent (B73) and six generations of crosses to a second, unrelated inbred (Mo17) (Table 7). The Chi square tests for the backcross to B73 and Mo17 did not deviate from expectations.

Assessment of possible toxicity

Introduction

Most of the studies were done using the insecticidally active trypsin-resistant core *E. coli* produced protein and not with plant-produced protein. The test proteins produced in *E. coli* are chemically and functionally the same as the plant-produced proteins (section 4.1.1).

Table 6. Segregation data of MON 810 progeny

Generation	Description	Actual	Expected	ChiSq
BCOF1 ¹	Derived from cross of R0 with an inbred line	44:47	45.5:45.5	0.044*
BC1F1 ²	Derived from cross of BCOF1 plants to the same inbred line used to cross the R0 plant	10:4	7:7	1.786*
BC1F2 progeny ³	Derived from cross of individual BCOF2 plants by a non-transgenic tested	69:181:77	81.75:163.5:81.75	4.138#

¹ Expressed as number of expressing plants: number of non-expressing plants based on ECB feeding assay.

² Expressed as number of expressing plants: number of non-expressing plants based on CryIA(b) ELISA.

³ Expressed as number of ear rows with homozygous number of expressing plants: number of ear rows with segregating plants: number of ear rows with homozygous susceptible plants based on ECB feeding assay.

* Not significant at $p=0.05$ (chi square = 3.94, 1df); # not significant at $p=0.05$ (chi square = 5.99, 2 df).

Table 7. Stability of gene transfer based on segregation data for backcross derivatives of MON 810 with two unrelated inbred lines (B73 and Mo17)

Generation ¹	Actual	Expected	Chi square
BC6F1 (B73)	8:13	10.5:10.5	0.762*
BC5F1 (Mo17)	11:11	11:11	0.045*

¹ Data expressed as number of expressing plants: number of non-expressing plants based on CryIA(b) ELISA.

* Not significant at $p=0.05$ (chi square = 3.84, 1 df).

Some of the food safety considerations are based on CryIA(b) characterization and digestive fate studies in simulated gastric and intestinal fluids.

Protein specificity

The CryIA(b) protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH, however, is solubilized by the alkaline gut of larval insects. The solubilized protein is then activated by the proteases in the insect gut, which diffuses through the peritrophic membrane to the midgut epithelium, binding to specific high affinity receptors on the surface. This paralyzes the gut due to changes in electrolytes and pH causing the insect to stop feeding and die.

There are no similar receptors for the protein delta-endotoxins of Bt species on the surface of mammalian intestinal cells, therefore mammals are not susceptible to these proteins. Also, absence of adverse effects in humans is supported by numerous reviews on the safety of Bt proteins.

Comparison to toxin databases

The CryIA(b) amino acid sequence was compared to known protein toxins. Similarity to a known toxin could trigger toxicological testing to address potential impact of the homology. B.t.k. HD-1 protein was compared to the amino acid sequences of 2632 toxins collected from

public domain genetic databases (GenBank, EMBL, PIR and Swiss Prot) for homology. The results confirm that the B.t.k. HD-1 protein is homologous to Bt insecticidal crystal proteins, but no amino acid homology was detected for other protein toxins. The closest match is shown in Figure 14.

Mouse acute oral gavage

An acute oral toxicity study (7 days) was done with albino mice using *E. coli* produced protein (converted to the trypsin resistant core) and tested for purity, potency and stability. The protein was administered by gavage to mice at targeted doses of 0, 400, 1000 and 4000 mg/kg. The highest dose represents the maximum hazard dose concept outlined in US Subdivision M Guidelines for biochemical pesticides. One group was dosed with 4000 mg/kg of bovine serum albumin (BSA) as a protein control.

No treatment related adverse effects were observed (Table 8) and no statistical differences in body weight measures or food consumption were seen. No differences were seen in gross pathology between the groups. The LC50 of the B.t.k HD-1 (truncated) protein in mice is greater than 4000 mg/kg with the NOEL set at that value.

Potential toxic contaminants

In response to queries about possible changes in contaminant levels due to the introduction of the

Table 8. Results of acute mouse gavage test with CryIA(b) protein

Test group	Weight pretest (g)	Weight at end (g)	Food consumption (mean g/day)
Vehicle control (buffer)	31.1 [25.5]	30.8 [25.1]	5.3 [6.4]
Control (BSA 4000*)	31.1 [25.4]	31.0 [24.7]	6.2 [7.3]
400 Bt protein	31.1 [25.4]	30.5 [25.2]	5.3 [8.0]
1000 Bt protein	31.0 [25.3]	31.1 [25.0]	5.3 [8.0]
4000 Bt protein	31.0 [25.5]	30.5 [25.5]	5.5 [8.0/7.4]

[females] / *mg/kg body weight

Table 9. Dissipation of B.t.k. HD-1 protein insecticide activity in simulated gastric fluids

B.t.k. HD-1 ($\mu\text{G}/\text{nL}$)	Tobacco budworm mortality		% change
	0	2 minutes	
0.75	29	3	-90
7.5	69	8	-88
75	94	24	-74

Table 10. Dissipation of B.t.k. HD-1 protein insecticide activity in simulated intestinal fluids

B.t.k. HD-1 ($\mu\text{G}/\text{nL}$)	Tobacco budworm mortality		% change
	0	19,5 hours	
0.75	26	25	-4
7.5	76	61	-20
75	100	90	-10

cryIA(b) gene, the company notes that for aflatoxins, tests with MON 810 from the 1993 field trial did not detect aflatoxins and therefore the test was not repeated.

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxanin-3-one) is not present in seeds of cereals and therefore does not pose a hazard to consumers of grain products.

Metabolic degradation in simulated gastric and intestinal fluids

Purified CryIA(b) protein (B.t.k HD-1 as expressed in *E. coli*) degrades rapidly in vitro using simulated digestive fluids. In the simulated gastric fluid, more than 90% of the protein degraded within two minutes, as detected by Western blot analysis (Figure 15). Lanes 6-11 are incubations at 0, 10, 20, 30, 60 and 120 seconds. Protein bioactivity detected using an insect bioassay also dissipated quickly with 74-90% of the added protein dissipated within two minutes (Table 9), the earliest time point measured. In a human stomach, approximately 50% of solid food empties to the intestines in two hours and liquids in about 25 minutes.

In the simulated intestinal fluid, the purified CryIA(b) protein did not degrade substantially after 19.5 hours as assessed by Western blot (Figure 16, lanes 8-11 are incubations at 0, 60 minutes, 4 hours and 19.5 hours) and insect assay (Table 10). This was anticipated since the tryptic core of Bt insecticidal proteins is known to be relatively resistant to serine proteases like trypsin, a key protease in intestinal fluid. The insect used for the insect assay studies was the tobacco budworm.

Assessment of possible allergenicity

Humans consume large quantities of proteins daily and allergenic reactions are rare. One factor to consider is whether the source of the gene being introduced into the plants is known to be allergenic. Bt does not have a history of causing allergy. "In over 30 years of commercial use, there have been no reports of allergenicity to Bt, including occupational allergies associated with manufacture of products containing Bt." Further, protein allergens need to be stable in peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response. Tests above show that the CryIA(b) protein does not survive under simulated gastric digestion. Another common factor of allergenic proteins is that they occur in high levels in the foods (e.g., allergens in milk, soybean, peanuts). This is not the case with the CryIA(b) protein which is present at approximately 0.19-0.39 $\mu\text{g}/\text{g}$ fresh weight of corn seed.

The company stated that Comparing sequences of amino acids to known allergens and gliadins is a useful first approximation of potential allergenicity or association with coeliac disease. A database of 219 protein sequences associated with allergy and coeliac disease assembled from genetic databases (GenBank, EMBL, PIR and Swiss Prot) was searched for sequences similar to B.t.k. HD-1 protein. "Most major ... food allergens have been reported and the important IgE

binding epitopes of many allergenic proteins have been mapped. The optimal peptide length for binding is between 8 and 12 amino acids. T-cell epitopes of allergenic proteins and peptide fragments appear to be at least 8 amino acids in length. Exact conservation of epitope sequences is observed in homologous allergens of disparate species. ... an immunologically relevant sequence comparison test for similarity ... is defined as a match of at least eight contiguous identical amino acids." No biologically significant homology nor immunological significant sequence similarities were found. The best match is shown in Figure 17. The results establish that B.t.k. HD-1 protein shares no significant similarity with known allergen or gliadin proteins.

In summary, the low levels of the protein in the corn, combined with the digestive lability and the lack of homology with known allergenic sequences indicate that this protein does not possess allergenic properties. Coupled with the history of use as a microbial control agent with no allergenic concerns, this indicates that there is no reason to believe that CryIA(b) should pose any significant allergenic risks for the consumption of products produced from insect-protected corn.

Compositional analyses of key Components, evaluation of metabolites, food processing and nutritional modification

Introduction

Nutritional data are important relative to dietary exposure to corn products. While little whole kernel or processed corn is directly consumed by humans, corn based food ingredients such as starch and corn oil are used.

Compositional data

Samples for composition analysis were collected at the same time and from the same six sites used for analysis of expression levels in corn grain for a one-time experiment.

Corn seed (grain) samples of MON 810 and the control MON 818 were analyzed for the following components and compared with available literature values:

- Proximates (moisture, protein, ash, fat, crude fibre)*
- Calories
- Carbohydrate
- Starch

- Fatty acid profile*
- Sugar profile
- Amino acid composition*
- Tocopherols*
- Phytic acid*
- Minerals (calcium, phosphorus)* as summarized in Table 11.

Parameters with an asterisk (*) are considered for feed assessments, while the other parameters (often derived from calculations) are not commonly considered.

Carbohydrates were not measured but deduced using the following calculation: % carbohydrates = $100\% - (\% \text{ protein} + \% \text{ fat} + \% \text{ ash} + \% \text{ moisture})$. Also, calories was a derived parameter using the following USDA approved calculation: $\text{calories (kcal/100g)} = (4 * \% \text{ protein}) + (9 * \% \text{ fat}) = (4 * \% \text{ carbohydrates})$.

There were no significant differences for the variables protein, fat, ash, carbohydrates, calories and moisture between the IP corn and its control and both were within the reported values from the literature.

MON 810 contained eight amino acids (cystine, tryptophan, histidine, phenylalanine, alanine, proline, serine and tyrosine), which were statistically different from the control. The mean values for six of these (all except cystine and histidine) are within literature ranges. Cystine and histidine for both lines were statistically higher than the literature range but within the range (1.9-2.3%) observed for two (MON 800/801) similar lines. The level of histidine for MON 810 (3.1%) is within the range of another previous study for two lines of similar genetic backgrounds.

For fatty acids and carbohydrates measured (starch, fructose, glucose, sucrose and phytic acid), no significant differences were found between the control and the IP lines. Crude fiber values in MON 810 grain (2.6%) were statistically different from MON 818, but both values were within the literature range (2.0-5.5%).

Tocopherols are naturally present in corn oil and have vitamin E potency. The gamma tocopherol is one-tenth as active as the alpha and is therefore not considered an important component of the corn grain. MON 810 values for the alpha and gamma tocopherols were statistically similar to the control but the beta tocopherol differs statistically from the control (Table 11).

For the minerals calcium and phosphorus, calcium levels in MON 810 were statistically higher than for MON 818, but within ranges reported for tests with MON 800/801. No statistical differences were found for phosphorus.

Table 11. Comparison of compositional analysis for MON 810 corn grain with control (MON 818) and literature values

<i>Component</i>	<i>MON 810¹ mean (range)²</i>	<i>MON 818 mean (range)²</i>	<i>Literature value⁴ mean (range) [MON 800/801 range]</i>
Proximate analysis			
Protein ³	13.1 (12.7-13.6)	12.8 (11.7-13.6)	9.5 (6.0-12.0) 12.3 (9.7-16.1) [11.2-13.6]
Fat	3.0 (2.6-3.3)	2.9 (2.6-3.2)	4.3 (3.1-5.7), 4.6 (2.9-6.1) [3.8-4.2]
Ash ³	1.6 (1.5-1.7)	1.5 (1.5-1.6)	1.4 (1.1-3.9) [1.5-1.8]
Carbohydrate ³	82.4 (81.8-82.9)	82.7 (81.7-83.8)	not reported [80.8-83.0]
Calories/100g	408.4 (407.0-410.1)	408.5 (406.0-410.1)	not reported [412.6-415.7]
Moisture %	12.4 (11.0-14.4)	12.0 (10.6-14.2)	16.0 (7-23) [13.0-15.8]
Amino acid composition - nutritionally essential⁵			
Methionine	1.7 (1.6-1.9)	1.7 (1.6-1.7)	1.0-2.1 [2.0-2.6]
Cystine	2.0* (1.9-2.1)	1.9 (1.8-2.0)	1.2-1.6 [1.9-2.3]
Lysine	2.8 (2.5-2.9)	2.8 (2.7-2.9)	2.0-3.8 [2.6-3.4]
Tryptophan	0.6* (0.5-0.7)	0.6 (0.4-0.6)	0.5-1.2 [0.5-0.6]
Threonine	3.9 (3.7-4.4)	3.8 (3.7-3.9)	2.9-3.9 [3.9-4.2]
Isoleucine	3.7 (3.3-4.1)	3.8 (3.6-4.0)	2.6-4.0 [3.5-3.8]
Histidine	3.1* (2.9-3.3)	2.9 (2.8-3.0)	2.0-2.8 [2.8-3.3]
Valine	4.5 (4.1-4.9)	4.6 (4.3-4.8)	2.1-5.2 [4.2-4.8]
Leucine	15.0 (14.1-16.7)	14.5 (13.8-15.0)	7.8-15.2 [13.6-14.5]
Arginine	4.5 (4.2-4.7)	4.5 (4.2-4.7)	2.9-5.9 [4.1-5.0]
Phenylalanine	5.6* (5.2-5.6)	5.4 (5.2-5.6)	2.9-5.7 [5.2-5.6]
Glycine	3.7 (3.4-4.0)	3.7 (3.5-3.8)	2.6-4.7 [3.4-4.2]
Amino acids - nonessential⁵			
Alanine	8.2* (7.8-8.9)	7.8 (7.5-8.0)	6.4-8.0 [7.8-8.2]
Aspartic acid	7.1 (6.4-8.2)	6.6 (6.3-6.8)	5.8-7.2 [6.7-7.3]
Glutamic acid	21.9 (20.4-24.4)	21.1 (20.1-21.6)	12.4-19.6 [19.9-21.4]
Proline	9.9* (9.7-10.5)	9.6 (9.4-9.8)	6.6-10.3 [9.0-9.4]
Serine	5.5* (5.3-5.9)	5.2 (5.1-5.4)	4.2-5.5 [5.5-6.1]
Tyrosine	4.4* (4.1-4.8)	4.0 (3.9-4.1)	2.9-4.7 [3.8-4.3]
Fatty acids⁶			
Palmitic (16:0)	10.5 (10.2-11.1)	10.5 (10.2-10.7)	7-19 [10.2-10.9]
Stearic (18:0)	1.9 (1.7-2.1)	1.8 (1.8-1.9)	1-3 [1.6-3.1]
Oleic (18:1)	23.2 (21.5-25.4)	22.8 (21.6-23.9)	20-46 [21.2-25.9]
Linoleic (18:2)	62.6 (59.5-64.7)	63.0 (61.8-64.6)	35-70 [58.9-65.0]
Linolenic (18:3)	0.8 (0.7-0.9)	0.9 (0.8-0.9)	0.8-2 [0.9-1.1]
Carbohydrates and fiber⁷			
Starch %	67.6 (65.3-69.7)	66.9 (64.6-69.0)	64-78.0 [63.7-71.5]
Crude fiber %	2.6* (2.5-2.8)	2.4 (2.3-2.5)	2.0-5.5 [1.98-2.61]
Sugars⁸			
Fructose	0.32 (0.23-0.35)	0.27 (0.22-0.40)	[0.47-0.96]
Glucose	0.44 (0.34-0.47)*	0.93 (0.79-1.12)	[0.47-1.03]
Sucrose	0.93 (0.79-1.12)	0.93 (0.68-1.11)	[0.40-0.94]
Phytic acid %	0.86 (0.81-0.91)	0.84 (0.79-0.91)	0.7-1.0 [0.45-0.57]
Tocopherols (mg/kg)			
Alpha	10.4 (9.7-11.3)	10.9 (9.9-12.1)	3.0-12.1 [7.3-12.3]
Beta	8.5* (8.1-9.2)	7.5 (7.0-7.9)	[7.9-10.7]
Gamma	20.2 (15.3-24.8)	21.6 (18.8-27.8)	[21.7-42.5]

(Continued)

Table 11. (cont.)

Component	MON 810 ¹ mean (range) ²	MON 818 mean (range) ²	Literature value ⁴ mean (range) [MON 800/801 range]
Inorganic components⁷			
Calcium %	0.0036* (0.0033-0.0039)	0.0033 (0.0029-0.0037)	0.01-0.1 [0.003-0.004]
Phosphorus %	0.358 (0.334-0.377)	0.348 (0.327-0.363)	0.26-0.75 [0.311-0.368]

¹ Values with * are statistically different from MON 818.

² Values reported are means of six samples from six sites. Ranges are the highest and lowest values across those sites.

³ Percent dry weight of samples.

⁴ Where there are more than one value, this indicates more than one published source.

⁵ Values for amino acids reported as percent of total protein.

⁶ Values for fatty acids are % total lipid. Other fatty acids were below the limit of detection of the assay.

⁷ Values on a dry weight basis.

⁸ Sugars measured as g/100g. Galactose, lactose and maltose were also measured, but values were below the limit of detection.

The company concluded, “Based on these data, it was concluded that there are no meaningful compositional differences between the IP corn lines ... and the control line, MON 818.”

Additionally, the company summarized its Nutritional analysis conclusions, “nutritional composition ... falls within the ranges of each nutrient measures for non-modified corn lines. It can be

concluded that there appears to be no meaningful effect on corn plant nutrient levels. Phenotype was not affected in any of the numerous ways that were measured. Of the vitamins and minerals measured there were no practical differences reported. In terms of nutritional composition, MON 810 may be considered to be substantially equivalent to regular corn.” ●