Canadian Food Inspection Agency
Agence canadienne d'inspection des aliments

PLANTS WITH NOVEL TRAITS

SUBMISSION EXAMPLE: DATA SUMMARY

Description of the regulatory process, framework, information requirements, and example data usually presented in support of environmental and livestock feed safety assessments for plants with novel traits.

The information provided has been summarized and only a subset of the data is provided. This should not be considered a complete submission but only an example to increase understanding of the regulatory process for plants with novel traits.

Canada

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1.0) Introduction

i) Scope and Background

Industry and producers say that biotechnology-derived agricultural products offer potential benefits such as helping control pests, mitigating crop losses and improving food and livestock feed nutrition. At the same time, the application of any new technology to agriculture, including biotechnology, raises questions—including the potential for effects to humans, livestock, or the environment.

To deal with these questions, government regulators carefully assess novel products derived through biotechnology. Safety assessments conducted by the CFIA are designed to obtain as much information as possible about the novel product in a comparative approach that was first described in an Organisation for Economic Co-operation and Development (OECD) publication in 1993. This OECD document was the result of some 60 experts from 19 countries who spent more than two years discussing how to assess the safety of genetically modified plants and foods This comparative approach was further endorsed by a Food and Agriculture Organization (FAO) World Health Organization (WHO) joint expert consultation in 1996 and again in 2000. World experts have concluded that this process serves to protect human, animal, and environmental health.

A PNT is a plant containing a trait not present in plants of the same species already existing as stable, cultivated populations in Canada, or is present at a level significantly outside the range of that trait in stable, cultivated populations of that plant species in Canada.

Before a PNT can be released into the environment and used as feed and food, it is evaluated for safety of end use, based on data collected from extensive testing. The data submitted has to be of the same high quality as that submitted to peer-reviewed science journals. For each new product, regulators must determine the following:

- potential effect of the product on human health
- potential impact of the product on livestock and livestock nutrition
- potential environmental impact of the product

More than one government department and agency is involved in the overall regulation of PNTs. The main Canadian regulators are the Canadian Food Inspection Agency (CFIA) and Health Canada. The CFIA's Plant Biosafety Office is responsible for regulating the environmental release in Canada of agricultural and horticultural crop plants with novel traits. The CFIA's Feed Section is responsible for regulating the use as livestock feed from PNTs. Health Canada is responsible for regulating food made from PNTs.

This document provides an overview of the principles and the scope of the process used to make assessment decisions on plants with novel traits. It also provides

examples of the type of data that is requested by the CFIA and generated by the applicant.

This summary document contains information from a submission by a developer. Full submissions range from several hundred pages up to several thousand pages, depending on the plant-trait combination and the required studies. If deficiencies or questions of clarification are found, as happened in this case, reviewers will ask for clarifications which typically result in the developer providing more data.

Examples of information and data provided in this summary are based on data submitted by applicants. Some of the data, and techniques employed to generate that data, may be superseded by newer techniques and methods. However, much of the interpretation will remain similar. The data and summary information is provided for illustration purposes and is not intended to be a definitive statement on methodology for collection or interpretation of data. It must be noted that certain information has been reduced to summaries and the data are only a subset of the data originally submitted.

The CFIA would like to acknowledge Monsanto Canada Inc. for making available data for the purposes of this document.

This document describes a PNT that has been assessed for unconfined environmental release and use as a livestock feed.

It should be noted that in practice a PNT with the potential to enter the food chain would also be assessed for food safety. Please note that Health Canada is responsible for assessing food safety.

ii) Regulatory Principles and Framework

In 1993, the federal government announced a framework for the regulation of biotechnology-derived products in Canada, called the *Federal Regulatory Framework for Biotechnology*. One of its principles was that existing legislation and regulatory bodies would be used to regulate biotechnology-derived products. This means that agricultural biotechnology-derived products are regulated using the same authority as agricultural products produced in more traditional ways, but using different administrative procedures. More information on agriculture-related acts and regulations that the CFIA administers and enforces is available at:

- http://www.inspection.gc.ca/english/reg/rege.shtml

The CFIA is responsible for the regulation of agricultural products derived through biotechnology, including plants, livestock feeds, fertilizers and veterinary biologics. The CFIA also conducts post-approval inspections and monitoring so that registered products continue to meet quality and safety standards. Health Canada is responsible for assessing the human health safety of products derived through biotechnology including foods, drugs, cosmetics, medical devices, veterinary drugs, and pest control

products. It is responsible for setting standards for safety of the food supply, including biotechnology-derived food products (novel foods). Environment Canada administers the *Canadian Environmental Protection Act, 1999* (CEPA, 1999) so that all new substances (including products derived through biotechnology) are assessed for their potential to harm human health or the environment. CEPA, 1999 currently has an "umbrella" type of regulatory authority over products of biotechnology, except where other departmental legislation and regulations include equivalent provisions. Both the *Feeds Act* and the *Seeds Act*, administered by the CFIA, are deemed to be equivalent to CEPA, 1999.

In many instances, a biotechnology-derived product is assessed under various legislation , so evaluation requires co-ordination across departments and agencies. The CFIA, Environment Canada, and Health Canada work together to assess the safety and environmental impact of these products.

Canada's agricultural biotechnology regulatory system is based on several fundamental regulatory principles, which influence biotechnology legislation and safety assessment guidelines. These are outlined below.

Science-based Approach

Safety assessment is based on the review of information about the biology of the organism, the technology used to create it and its effects on the environment and living organisms (including humans). Scientists from the CFIA and Health Canada are responsible for critically reviewing the data collected from laboratory and field experiments submitted by the applicant.

Product-based Approach

Canada regulates biotechnology-derived products based on their function and use, not on how they are made. The decision to follow this route was based in part on the fact that Canada had several existing product-based acts (e.g. for feeds, fertilizers, and seeds). Regulators saw new biotechnology methods (e.g. recombinant DNA techniques) as another means to produce new lines of the same family of products. Biotechnology applications were, therefore, added, where appropriate, to existing acts and guidelines.

Familiarity and Substantial Equivalence

"Familiarity" means using knowledge about the characteristics of a species and practical experience with that species to make regulatory decisions. It has been used both to determine whether a product should be reviewed, and as part of the review process (e.g. comparing a modified organism with its parents or with related organisms). Familiarity led to the concept of "substantial equivalence", which is used in Canada and other jurisdictions. Substantial equivalence is used to determine, in part, how a new

product should be regulated. If a modified plant or product is considered substantially equivalent to an unmodified counterpart plant or product, it is regulated in a similar way. If it is not substantially equivalent, it may require further evaluation or a different approach to determine its safety.

Case-by-case

Each new product is treated as a unique case because each different plant-trait combination may pose unique risks.

Step-wise Approach

Products are assessed step-by-step throughout the development process. This assessment approach progresses “from the laboratory to the growth chamber and greenhouse, to limited field testing and finally to large-scale field testing.” (OECD) The concept is to build on and refine the information based on the steps conducted before.

Transparency

Openness is key to an effective regulatory framework. Stakeholders from government, academia, industry and the public are consulted during the development of new regulatory requirements. Regulators also inform the public of decisions made using assessment procedures in decision documents that are available from the CFIA at:

- http://www.inspection.gc.ca/english/plaveg/pbo/dde.shtml

Precautionary Approach

Many national and international regulatory bodies have adopted a precautionary approach as a key principle of their regulatory systems. The application of precaution recognizes that the absence of full scientific certainty should not be used as a reason to postpone decisions where there is a risk of serious or irreversible harm. It is characterized by three basic precepts: the need for a decision, a risk of serious or irreversible harm, and a lack of full scientific certainty.

Canada has a long-standing history of using this precautionary approach in the federal regulatory system. The regulatory system in this regard is determined by the applicable provisions of federal law, binding federal-provincial agreements, and international agreements to which Canada is a party.

Canada's *Framework for the Application of Precaution in Science-Based Decision Making About Risk* is available on the Privy Council Office web site at:

- http://www.pco-bcp.gc.ca

Harmonization

Canada participates in the Organisation for Economic Co-operation and Development (OECD) Working Group on Harmonization of Regulatory Oversight in Biotechnology and the Taskforce for the Safety of Novel Foods and Feeds. The main focus of the work done by these groups is to develop consistent regulations for biotechnology among member countries, while avoiding trade barriers. Canada is also interested in bilateral initiatives. For example, Canada and the US have harmonized their data requirements for molecular characterization components of the regulatory review process for transgenic plants. For more information, see the Canada and United States Bilateral Agreement on Agricultural Biotechnology, at:

- http://www.inspection.gc.ca/english/plaveg/bio/usda/cdausbilate.shtml

The Cartagena Protocol on Biosafety also outlines international harmonization procedures for “transboundary movement of living modified organisms resulting from modern biotechnology” and their potentially adverse effects on biological diversity and human health. Annex III of the document sets out the objective, use of, general principles, methodology and points to consider for risk assessment. See:

- http://www.biodiv.org/biosafety/articles.asp?lg=0&a=bsp-a3

iii) Plants With Novel Traits (PNTs)

A plant with a novel trait (PNT) is a plant containing a trait not present in plants of the same species already existing as stable, cultivated populations in Canada, or is present at a level significantly outside the range of that trait in stable, cultivated populations of that plant species in Canada. They can be produced by conventional breeding, mutagenesis or recombinant DNA techniques such as genetic engineering. To date, over 50 PNTs have been approved for environmental release and/or use as livestock feed in Canada. For the latest information, and links to detailed descriptions of each of these approved PNTs, see:

- http://www.inspection.gc.ca/english/plaveg/bio/pntvcne.shtml

There are several stages in the development of a PNT. In most cases, novel plants, depending on their history and intended use, go step-by-step through the stages of containment, confined field trials and then application for unconfined environmental release, use as a livestock feed, and food safety approval. The purpose of this document is to illustrate two components of this final stage: the safety assessment for environmental release and livestock feed use. Food safety assessment is the responsibility of Health Canada, and is beyond the scope of this document (more information can be found on the Health Canada web site, at:

- http://www.hc-sc.gc.ca/english/protection/biotech/index.htm

Most PNTs are designed with the intention of unconfined release and commercialization. However, in the early stages of developing a new PNT, plants are grown under contained conditions in a laboratory growth chamber or greenhouse before

undergoing confined research field trials. Field trials yield specific information that developers must collect and submit to the CFIA when applying for unconfined release of the PNT.

Before PNTs can be released into the environment in Canada, or used as livestock feed or human food, several authorizations must be granted. The plant must be approved for full environmental release by the CFIA's Plant Biosafety Office, for livestock feed use by the CFIA's Feed Section and for food use by Health Canada. Assessment at this stage identifies potential adverse impacts associated with the release of the PNT. Unconfined release of the novel plant may be permitted with special conditions. For example, some PNTs may only be grown in some regions of Canada. Also, insect-resistance management plans must be followed if the PNT is resistant to pests (e.g. Bt corn). More information on insect-resistance management can be found at:

- http://www.inspection.gc.ca/english/plaveg/bio/bt/bacthue.shtml

If the PNT is to be used as a livestock feed, the Feed Section of CFIA will conduct a feed safety assessment. The modified plants are assessed for potential impacts on livestock nutrition and impacts on livestock and workers/by-standers. The assessments take into consideration the intended effect of the modification, as well as unintended changes that may have occurred.

The CFIA provides documents called regulatory directives that elaborate on and assist a person in determining how to comply with the various Acts and Regulations for products of biotechnology. Here are some examples:

Regulatory Directive 94-08: *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits*, at:

- http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml

Regulatory Directive 95-03: *Guidelines for the Assessment of Novel Feeds: Plant Sources*, at:

- http://www.inspection.gc.ca/english/anima/feebet/dir9503e.shtml

Regulatory Directive 2000-7: *Guidelines for the Environmental Release of Plants with Novel Traits Within Confined Field Trials in Canada*, at:

- http://www.inspection.gc.ca/english/plaveg/bio/dir/dir0007e.shtml

Regulatory Directive 96-13: *Import Permit Requirements for Plants with Novel Traits and Their Products*, at:

- http://www.inspection.gc.ca/english/plaveg/protect/dir/d-96-13e.shtml

iv) Data Requirements for Environmental Release and Livestock Feed Approvals

To assess new PNTs for environmental release and for feed safety, CFIA regulators require detailed data about each modified plant. All data must be rigorous and scientifically sound. If it is incomplete or inadequate, the product developer must address these issues before the assessment process can continue. Data requirements may vary. For example, crops such as canola that can outcross with wild relatives require more environmental data than crops such as corn that have no wild relatives in Canada.

Information required for the safety assessments can include core data on the identification and classification of the plant, the method of genetic modification, detailed molecular analyses, compositional and nutritional data, and environmental data (effects and fate). In some cases, government evaluators will also contract studies or convene groups of experts to gather further information about specific types of products, including research about their potential interaction with the environment.

A description of the data required during environmental safety or feed safety assessments follows. Detailed information is available in Regulatory Directive 94-08: *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits,* at:

- http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml

and Regulatory Directive 95-03: *Guidelines for the Assessment of Novel Feeds: Plant Sources*, at:

- http://www.inspection.gc.ca/english/anima/feebet/dir9503e.shtml

The applicant is required to submit to the CFIA as much data as required to address evaluation criteria. (Note that food safety assessments are conducted by Health Canada, and data associated with these assessments is not included in this document.) Submissions include detailed information on the plant that has been modified, as well as of pattern of its typical use and the intended use of the plant with novel traits. For illustration, these are categorized and described briefly below.

Crop Biology

For most practical purposes the crop that has been modified usually comprises a species that is widely grown within Canada (or used commercially) and hence there is usually a significant level of background information on the biology and husbandry of the crop in question, as well as use patterns. For a crop that has been modified by the addition of a novel trait, the information provided includes bedrock information on the plant and its particular characteristics. Much of this information is now defined for each of the major crops through crop-specific companion documents. See:

- http://www.inspection.gc.ca/english/plaveg/bio/dir/biodoce.shtml

In addition, where an “unfamiliar” use is contemplated, a detailed explanation of that use is required. Typical information required includes:

- name and taxonomy
- history or pedigree, or other information about the organism that defines its unique character
- how it will be used (the application)
- history of use as an animal feed

Genetic Modification

Extensive information is required that describes the genetic modification, the techniques used, and the characterization of the modification event. Many different techniques can be used to develop a novel plant. These include traditional breeding, recombinant DNA techniques (such as genetic engineering), mutagenesis, transformation and cell fusion. Modification data should include the following elements:

- description of the modification technique used
- identification of all genetic material potentially delivered to the host plant
- information about the process and the DNA used to modify the plant (e.g. plasmids, identity, expected function) including intermediaries (e.g. bacteria)
- description of the DNA to be introduced (e.g. characterization, size, location and orientation in the final vector/construct, function)

Basic techniques of recombinant DNA and short primers on basic recombinant techniques can be found at:

- http://www.genome.ou.edu/protocol_book/protocol_index.html
- http://www.cas.vanderbilt.edu/bsci111b/recom-1/supplemental.htm

and links therein.

For plants modified by non-recombinant processes, a detailed explanation of the process used is required. These other processes may require additional technical information to be submitted so that the CFIA has a complete description of the methods used.

For procedures that involve the use of plant transformation, a detailed explanation, including scientific references of the process, must be provided. A brief overview of details on crop transformation can be found at

- http://opbs.okstate.edu/~melcher/MG/MGW4/MG4373.html
- http://www.nuffieldbioethics.org/publications/gmcrops/rep0000000086.asp

Plants with a novel trait that are submitted for regulatory review undergo an extensive molecular characterization. This includes characterization of the gene(s), following insertion into the plant genome, and the integrity of the gene and its activity under typical conditions.

A PNT can have several novel traits. Novel traits may result from a gene being inserted into a plant. For each gene inserted into a novel plant product, including marker genes, the following data is required:

- how the gene is expressed (e.g. stability, tissue-specific expression, expression levels, etc.)
- description of the breakdown products
- known toxicity or interaction with other organisms
- allergenicity potential

PNTs are characterized by their equivalence to unmodified plants of the same variety; the product of the gene itself is characterized in the plant, and any potential changes in the plant physiology, biology or biochemistry are noted. In the case of an inserted gene, the fate of the novel gene product is studied throughout the life cycle of the plant, by observing its expression and its catabolism.

A significant amount of this information is presented in the form of molecular biology data that include such techniques as Southern Blots, Northern Blots, Western Blots, and ELISA, which are able to detect single genes, quantify expression of a single gene, and identify protein products. For a review of various analytical techniques, an understanding of how these techniques are interpreted and used to generate data, please refer to the following sites:

- http://www.botany.uwc.ac.za/mirrors/MIT-bio/bio/rdna/rdna.html
- http://www.mcb.uct.ac.za/manual/MolBiolManual.htm
- http://www.dnalc.org/resources/BiologyAnimationLibrary.htm
- http://www.lsic.ucla.edu/ls3/tutorials/

Environment

Modified plants intended for environmental release are examined for interactions with, and impact on, the ecosystems in which they will be used. Data can be a combination of literature on the plant (e.g. flowering period, seed production) as well as test data from the laboratory or greenhouse or from field testing (e.g. potential to become a weed or plant pest).

The following criteria are used to assess the interactions of PNTs in the environment:

- Does the plant have the potential to become a weed of agriculture or to be invasive of natural habitats?
- Is there potential for gene flow to wild relatives whose hybrid offspring may become more weedy or invasive?
- Does the plant have the potential to become a plant pest?
- Is there a potential impact on non-target organisms?
- Is there a potential impact on biodiversity?
- If the plant expresses an insecticide, is an insect resistance management plan in place?

This information is often collected through multiple years of testing under various levels of environmental exposure. For example, plants modified to be resistant to insect or microbial pests are not only evaluated for their resistance, but also evaluated for their

effect on the overall environment, including soil environment and impact on non-target pests. The development of data that accurately defines the environmental impacts of a plant with novel traits is a crucial aspect of the assessment process. At present, there is a large degree of understanding of the environmental impact of certain traits, whereas other traits that may be more complex in nature will require ongoing work and development in order to fully understand the environmental impact.

In addition to developing data that describes the interaction of the modified plant with the environment, proponents of a PNT submit data related to agronomic properties and other practical aspects of cultivation. Examples include potential for the plant to become a pest or a weed; an assessment of the PNT relative to the unmodified plants in terms of reproduction, survival, adaptation, stress response, gene flow through outcrossing; and impact on non target organisms.

Livestock feed

Data must be submitted to determine the impact of the modified plant on livestock and livestock nutrition, and the potential impact on workers/by-standers who may come in contact with the feed.

Nutritional information is required for PNTs that will be used in livestock feed. Data requirements may include: measurements of basic nutritional components, protein fat and fibre; compositional data on total lipids, the carbohydrate fraction, minerals and antinutritional components such as phytates, or trypsin inhibitors.

The dietary exposure to the novel feed is a consideration in the safety assessment. Feeds that are present in low levels in a complete diet may be of less concern than those intended to be significant components of the diet.

Toxicity and allergenicity data for PNTs that will be used in livestock feed are derived from product information and laboratory studies. The information required depends on the characteristics of the modified plant and the novel substances it expresses. Information can include data on the following:

- concentration of the novel substance in edible parts
- comparison of amino acid sequences and similarities to known toxins
- stability to heat or processing methods
- degradation in representative gastric and intestinal model systems
- allergenic potential of gene products
- toxic potential of gene products

The potential for allergenic response in humans who would be exposed to the livestock feed, in their work environment for instance, would be considered on the basis of the history of the host and donor organisms and the novel traits introduced.

Techniques

It is noteworthy that there are continual changes to the analytical tools and techniques that are used to collect data. Much of the data presented in this study to illustrate the assessment process was submitted a number of years ago. Thus, new data submitted in support of a new submission may be collected and presented in a different form. Although new techniques can be used to generate data, the essential interpretation of the data will follow a similar path to that described here.

2.0) The Data Summary

This summary illustrates data used for the safety assessment of a plant with a novel trait intended for unconfined environmental release and use as a livestock feed, as conducted by the CFIA. Please note that Health Canada is responsible for food safety assessments, and information on this aspect of biotechnology regulation can be found at:

- http://www.hc-sc.gc.ca/english/protection/biotech/index.htm

The data summary is based on the assessment of corn modified to be insect tolerant through the insertion and expression of a *Bacillus thuringiensis* (Bt) gene. This information, supplied by Monsanto Canada Inc., illustrates the development of a PNT by the use of biolistic transformation to incorporate a single copy of a Bt gene into the corn genome.

For illustrative purposes this document will rely on information submitted for the safety assessment of this genetically modified insect resistant corn. The following pages will provide examples of the data submitted, the nature of data and the interpretation of data in the following areas:

- description of the novel trait
- characterization of the genetic modification
- environmental assessment
- livestock feed assessment

The information that is required and assessed under these sections is summarized. Within each of these different areas, the nature of the information itself, and the techniques used to generate the data, are described in detail.

The reader is referred to the CFIA web site for detailed information and answers to general questions on the regulatory process:

- http://www.inspection.gc.ca/english/plaveg/pbo/pbobbve.shtml

3.0) Data Summary Details

i) Description and Assessment of the Novel Trait

The information requirements related to the description and assessment of the novel trait found in regulatory directives Dir94-08 *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits,* and Dir95-03 *Guidelines for the Assessment of Novel Feeds: Plant Sources* are focused on the molecular biology of the trait and the mechanisms that have been employed in the modification of the plant. The directives share a number of requirements, although there are requirements that are unique to the feed assessment. This section outlines those requirements.

Under Dir94-08, the following PNT information is collected:

- plant taxonomy (scientific and popular name)
- common designation given to the PNT, including all synonyms
- pedigree information of the PNT (including any relationship to a previously assessed
- PNT)
- the use of the PNT

Subsection information includes:

- description of the novel gene product(s) conferring the novel traits
- methods used to introduce the novel traits

If recombinant DNA techniques were used, a map of each genetic construct and details of the genes is also required, including:

- other marker genes
- regulatory sequences
- any other DNA sequences
- vector name
- characteristics and features

Other information includes the parental genome with the genetic modification (for allopolyploid PNT), number of generations removed from the original modification and information on expression and stability of expression.

Other information required includes: information on any gene products that are tissue-specific or expressed during a specific developmental stage or upon induction. The activity of the gene products, breakdown products and by-products in the host plant are also required, as well as any changes to existing metabolic pathways observed or anticipated. Toxicity data required includes: potential toxicity to known or potential predators, grazers, parasites, pathogens, competitors and symbionts; potential for adverse human health effects and most likely route of human exposure to the gene products, breakdown products and by-products.

Additional data required for feed assessments, as described in Dir95-03, that is distinct from that described above includes:

- a determination of the expression level of the gene in all plant parts that may be fed to livestock
- the activity of the gene product(s), breakdown products and byproducts in the host plant, and any resulting changes to existing metabolic pathways (including altered accumulation and storage patterns)
- the likelihood of exposure to humans and livestock, including the estimated level and most likely route of exposure to the gene products, breakdown products, and byproducts
- the potential for adverse health effects to humans and livestock, including exposure to toxins and anti-nutritional factors, and irritation and allergenicity to humans (i.e. in terms of occupational exposure)

If the modification causes the production of novel proteinaceous material, similarity to products from traditional sources should be described, where appropriate. Where genetic modifications alter the expression of a traditional plant constituent, sufficient information on the anabolic or catabolic pathways should be provided to enable an assessment of possible secondary effects on related pathways and metabolite production. Any transformation markers used in the development of the variety must be identified. Where there are secondary effects on the biochemistry, physiology and secondary metabolism of the host plant, these should be characterized. Information should be provided on the consequences to the final plant.

The actual assessment of the novel trait will be highly dependent on the nature of the trait itself. For traits that have a history of safe use, have been well described and understood, the assessment may be relatively straightforward. An example of this is the Bt gene. A large body of data has been accumulated on many aspects of Bt use and this provides a convenient starting point to assess the PNT. There are differences of expression of a Bt gene inserted into plants versus the traditional use of applied Bt, but familiarity with Bt products provides a strong point of comparison. However, it must be understood that only a few traits exist that have a long history of safe use. Every newly engineered trait will require the development of an appropriate data package for assessment.

Example data

As part of the assessment, data is submitted on the host plant itself (see Appendix A: Crop Species), and the nature of the trait being introduced. In the example using Bt, the history of safe use and the familiarity of the novel trait provide useful background for an assessment review (see Appendix B: Novel Trait Familiarity). In assessing the novel trait it is important to develop an understanding of the mode of action. The ability to make informed choices regarding the essential tests that are required to prove that the novel trait will provide the intended effect is dependant on this understanding. For example, if the novel trait encodes an insect resistance, adequate background information concerning efficacy and the mode of action of the insect resistance protein that is required to form the backdrop over which assessment is conducted at the whole plant level. Similarly, for a trait encoding a herbicide tolerance, the mode of action of the herbicide, the potential side effects, as well as the activity of the tolerance gene itself has to be well understood in order to devise mechanisms to allow for critical assessment of the functionality of this novel trait within the whole plant and under a larger environmental release.

The nature of the trait DNA construct is also essential. Although there is significant body of scientific literature describing various genes and DNA components, the applicant must provide a detailed overview of the genetic construct and its components (see Appendix C: Genetic Constructs). If there has been a history of use of these particular genetic elements within the crop species that is being assessed, background information should also be made available.

The expression and stability of the novel trait and the pedigree of the PNT are also important considerations. Representative data explaining these features of the PNT are described (Appendix D: Expression).

ii) Characterization of the Genetic Modification

A primary package of information is directed to the description of the novel trait and the genetic constructs (or methods) used to confer the novel trait. A new recombinant genetic element typically comprises a promoter (or region that controls the expression of DNA, adjacent to the coding region), a coding region (which can comprise a protein coding sequence or a sequence that is transcribed into RNA that can affect the function of a plant cell), and a termination sequence that defines the end point of the transcription of the DNA. This combination of three elements (promoter, coding region, and termination) is referred to as a gene. Genes are typically propagated in plasmid cloning vectors, which allow their manipulation, sequencing and other types of modification in a convenient fashion. The gene itself can be removed as an intact fragment and moved to either a transformation vector used by Agrobacterium or incorporated into a plasmid that is then used for transformation via the biolistics process.

Regulatory Directives 94-08 and 95-03 tell applicants that they also have the duty to provide any information about the modification that may include novel gene products conferring the novel traits, and methods used to introduce the novel traits. If the modification was achieved through recombinant DNA techniques, details of the genetic construct and mode of transformation are required. Additional information is required on the analysis of the genetic modification within the PNT itself. This includes information on novel trait gene copy number and methods used to introduce the modification into the commercial variety (e.g. information on pedigree). The description of the genetic modification itself is the main element for this portion of the data.

The data that allows assessment of the genetic modification includes analysis to identify the number of copies and the configuration of the inserted gene and that there are no partial or fragmented copies of the gene are present in other regions of the plant genome. Data on the transformation process, the methods used to select the plant carrying the genetic modification, and any other pertinent information in the development of the PNT has to be given.

Example data

A detailed characterization of the gene being inserted and the plasmid vector that carries this gene is required. In the example provided, the gene is contained in a plasmid cloning vector that is then used for transformation via the biolistics process (see Appendix E: Transformation Process). Detailed restriction mapping or PCR characterization of the genetic insert demonstrates that the gene has been inserted as originally intended.

Southern blots in this summary (see Appendix F: Molecular Characterization) demonstrates that the PNT only contains the gene encoding the novel trait, and not any selection genes.

Data is also supplied that illustrates the gene encoding the novel trait expresses properly and produces a protein that is the same as predicted and possess the appropriate characteristics and properties (see Appendix G: Trait Integrity).

Due to the low expression of this protein in the modified corn, it was necessary to produce the protein in a bacterial expression system to obtain enough material to perform various toxicity studies needed to fulfill regulatory requirements. The equivalence of bacterial and plant produced proteins is demonstrated in Appendix G.

iii) Criteria for Environmental Assessment

The overall purpose of the data collected in this portion of the assessment is to determine:

- potential of the PNT to become a weed of agriculture or be invasive of natural habitats
- potential for gene-flow to wild relatives whose hybrid offspring may become more weedy or more invasive
- potential for the PNT to become a plant pest
- potential impact of the PNT or its gene products on non-target species, including humans
- potential impact on biodiversity

The data package considered in the environmental safety assessment of PNTs includes identification of potential concerns, required information, and procedures to assess potential environmental impacts associated with unconfined release of PNTs. The assessment criteria are designed to be used in conjunction with species-specific companion documents, that describe the biology of the species to which the modified plant belongs, including details of other life forms with which it interacts.

The information that is to be assessed includes:

<u>Biology and Interactions of the PNT</u>
Identification of anticipated or observed differences between the PNT and the unmodified form or a named counterpart (closely related genotype, or with a range of named counterparts of similar plant type). This is done to determine whether there are significantly different or altered interactions with other life forms, resulting from the PNT's novel gene products, that could potentially cause the PNT to become a weed of agriculture, become invasive of natural habitats, or be otherwise harmful to the environment.

Specific information includes:

- reproductive and survival biology
- adaptations to stress factors (for biotic stress factors, this means identifying those life forms with which the PNT interacts differently)
- biochemistry of novel gene products that are known to be toxic
- a description of the likelihood that levels of exposure of consumers and soil micro flora and fauna symbionts may be altered

Residual studies may be conducted to determine macro changes, and additional information on relative phenotypic expression of the PNT is also required.

<u>Interactions of the PNT</u>
Information in this section should clearly describe the methods used to obtain the information, together with bibliographic references, including numbered patents, where these are appropriate. Information will include plant characteristics in the environment

such as:

- habit (annual, biennial, perennial)
- vegetative vigor (biomass)
- overwintering capacity (plant counts)
- flowering period
- time to maturity
- seed production
- dormancy
- reproductive characteristics
- outcross frequency within species
- cross-pollination vectors
- fertility (male and female)
- self compatibility
- asexual modes of dispersal
- stress adaptations
- residual effects
- composition
- endogenous toxins
- non-endogenous
- any other observations

Information collected regarding agricultural or silvicultural practices will include:

- proposed release sites for the PNT, if the modification result in the PNT being grown outside of the normal geographic production area or outside of the usual habitat (e.g. cultivated agricultural lands) for the species
- information on deviations in cultivation practices and control and mitigation procedures
- post-harvest procedures, including procedures for disposal of remaining plant matter

Additional information may be required regarding the potential environmental effects such as development of weediness or directly becoming a pest.

Example data

The type of data reviewed includes information on the biology of the PNT under typical environmental exposure (see Appendix H: Agronomic Data), including agronomic data and general plant characteristics.

Additional data will include information on interactions of the PNT, such as the reproductive and survival biology, adaptation to stress, and gene flow data (see Appendix I: Environmental Interactions). This data is usually compared to the same data for a plant of the same species or equivalent variety.

Data that describes the interaction of the PNT with non-target organisms, effects on biodiversity and environmental fate of the PNT product is also provided (see Appendix

J: Trait Specificity).

Lastly, a discussion regarding the impact on production (cultivation) practices is provided (see Appendix K: Cultivation). For insect resistant crops, insect resistance management guidelines must also be followed.

iv) Criteria for Livestock Feed Assessment

The primary consideration in a livestock feed assessment is the impact of the PNT, or product of the PNT, on livestock and farm workers. A wide variety of modifications are possible through both traditional breeding and genetic engineering. These modifications may unintentionally introduce toxic compounds, cause secondary effects, and cause changes in nutritional and safety characteristics of the feed with potential safety concerns. To ensure that these concerns are addressed, there must be a clear understanding of the novel trait(s), which is partly determined by previously considered information. A guiding principle in the feed safety assessment is the comparison of molecular, compositional and nutritional data of products derived from the modified plant to those of traditional counterparts. Where similarity or degree of equivalence cannot be established, a more extensive feed safety and efficacy assessment is necessary.

Detailed data characterization should be generated with genetically stable, converted lines that are representative of the final feed product. Sufficient data should be submitted to characterize the modified plant and allow for a comparison with the conventional or unmodified counterpart. Most of the questions regarding characterization of the modified plant can be addressed by data that may have been generated in the developmental stage. The presence and level of toxic or anti-nutritional compounds from novel plants developed from parents or vectors known to express these substances are of special concern. Relevant information on the donor and host organisms should be considered and include a critical assessment of the ability of both donor and host organisms to produce potentially toxic compounds, available toxicology data, history of safe use of livestock feeds from the host plant and related varieties used in the development of the modified plant.

Newly expressed material, which is either introduced or modified native material, should be assessed to provide the following information:

- the gene product(s)
- breakdown products, byproducts and their metabolic pathways
- a determination of the expression level of the gene in all plant parts that may be fed to livestock
- the activity of the gene product(s)
- breakdown products and byproducts in the host plant, and any resulting changes to existing metabolic pathways (including altered accumulation and storage patterns)
- the likelihood of exposure to humans and livestock, including the estimated level and most likely route of exposure to the gene products, breakdown products, and

byproducts
- the potential for adverse health effects to humans and livestock, including exposure to toxins and anti-nutritional factors, and irritation and allergenicity to humans (i.e. in terms of occupational exposure).

Where the result of the modification is the production of novel proteinaceous material, similarity to products from traditional sources should be described, where appropriate. Where genetic modifications alter the expression of a traditional plant constituent, sufficient information on the anabolic or catabolic pathways should be provided to enable an assessment of possible secondary effects on related pathways and metabolite production.

A determination of the potential for any introduced antibiotic resistance gene, typically used as a selectable marker during plant transformation, to have an effect on antibiotics added to feed and/ or the inactivation of these added antibiotics during storage must be completed.

In addition to this information, submissions must be accompanied by adequate data to demonstrate that there is no significant change in the nutritional composition of the livestock feed product(s), when compared to those originating from a presently accepted source. If the composition of the proposed feed ingredient is judged not to be substantially equivalent to that of an approved feed ingredient, additional nutritional data will be required. The required data will be a function of the nature and degree of difference with respect to the feed ingredient from an accepted source. The following analyses must be conducted on all feeds from plants with novel traits:
- crude protein
- crude fat
- fibre, any of crude fibre, ADF (Acid Detergent Fibre) or NDF (Neutral Detergent Fibre)

A statistical comparison of these nutrient compositions should be done and both the raw data and computer printouts of statistical analysis must accompany the submission.

The applicant must provide a description of the feed ingredient(s) and detailed information on processing methods and proposed use(s). Additional information may be required such as:
- content of true protein, non-protein nitrogenous material, and amino acid profile
- quantitative and qualitative composition of total lipids and complete fatty acid profile
- composition of the carbohydrate fraction; qualitative and quantitative composition of vitamins (i.e. complete vitamin analysis)
- mineral analysis
- presence of naturally occurring or adventitious antinutritional factors that could reasonably be expected to be present (e.g. phytates, trypsin inhibitors, alkaloids, pigments, etc.)
- dietary exposure, toxicology data and feed trial information may also be submitted

Example data

The nature of the data submitted in this portion of the assessment will include information on the protein expressed in a PNT for toxicity (see Appendix L: Toxicity and Allergenicity), feeding studies (see Appendix M: Feeding), and compositional data (see Appendix N: Compositional Data).

Compositional data must be generated for the PNT and must be compared to an unmodified plant grown at the same location (see Appendix N: Compositional Data). Consensus documents on key nutrients and toxins of various crop plants are available from the Organisation for Economic Co-operation and Development, found at:

- www.oecd.org/ehs

The compositional data should be generated using sound experimental design principles and recognized analytical methods such as those published by AOAC International, at:

- http://www.aoac.org/pubs/oma_revised.htm

Comparison of nutritional composition of the PNT to published nutrient databases such as the National Research Council (United States) or Agricultural Research Council (United Kingdom) may also be helpful.

4.0) New Information Requirements

The CFIA may develop new information requirements, and does review new product information that has come to light. In addition, on a case-by-case basis, the CFIA may conduct auditing and additional studies pertaining to biotechnology issues of interest. In this manner, CFIA scientists build a more informed regulatory knowledge base and stay in touch with any evolving changes in science. For example, the CFIA has commissioned studies on the effects of Bt corn on insects , and herbicide resistance management for PNTs.

In addition, the CFIA considers additional scientific information as it comes to light and reviews this information in the context of previous safety assessments. This has taken place when the developer of a PNT brings new scientific findings to CFIA's attention. In some instances approvals may continue, or the government has the authority to place restrictions on the use of the PNT or cancel the approval. For example, see:

- http://www.inspection.gc.ca/english/sci/biotech/tech/greenrounde.shtml

The data used in this submission example and data summary are considered current, accurate, and not under any additional review.

5.0.) Regulatory Decisions

A number of regulatory decision documents are available that provide a discussion of the data and how an assessment decision was made. The decision documents encompass a number of different plant species that represent a broad range of PNTs. They can be found at:

- http://www.inspection.gc.ca/english/plaveg/pbo/dde.shtml

Health Canada assesses food safety for novel foods or foods derived from PNTs. For a review of those safety assessments, the following link provides access to representative decisions:

- http://www.hc-sc.gc.ca/english/protection/novel_foods.html

Other regulatory decisions affect the cultivation of certain PNTs, especially insect resistant management strategies for crops that carry insect resistance. Information on such strategies for Bt corn can be found at:

- http://www.inspection.gc.ca/english/plaveg/bio/bt/btcormai1e.shtml

To see the decision document for MON810 Bt corn, go to:

- http://www.inspection.gc.ca/english/plaveg/bio/dd/dd9719e.shtml

Appendix A: Crop Species

Corn and its Uses

Zea mays L. (corn, or 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. It 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 has many uses. It is used as a staple food in many parts of the world. In its derived forms, it is used in such things as starch, alcohol, and oil. It is also used for livestock feed and for production of ethanol (a renewable fuel).

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 livestock feed because they are field corn and not sweet corn. For more information, see the biology companion document *Regulatory Directive Dir94-11: The Biology of Zea mays* L. (Corn/Maize), found at:

- http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml

Insect resistant (protected) or Bt corn variety MON810 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 company further describes the variety and its history, “Line MON810 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.”

MON810 is a field corn, not a sweet corn, and while it is intended primarily as a livestock feed, some human food uses occur for field corn. For example, MON810 may be used either dry or wet milled in processed corn products for humans. No new use of MON810 is expected; it will be used in the same way as existing field corn hybrids.

Appendix B: Novel Trait Familiarity

Bacillus thuringiensis (Bt) and its History

The cryIA(b) gene inserted into MON810 originates from a *Bacillus thuringiensis var. kurstaki*. *Bacillus thuringiensis* (or Bt) species are spore-forming, gram-positive bacteria that produce a crystal with insecticidal properties and 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 San Diego* and *tenebrionis* strains for coleoptera (Colorado potato beetle, elm leaf beetle, yellow mealworm)
- *Bt kurstaki, thuringiensis, sotto* and *aizawai* strains for lepidoptera (corn borer, tomato hornworms, gypsy moth, cabbage looper, tobacco budworm, cotton bollworm).

In Bt, the delta endotoxin crystals are produced when the bacterium sporulates. To be active, the insect must ingest the protein. While the protein is insoluble at neutral or acidic pHs, 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 end 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 the specific 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 insect protected (IP) corn is identical to that produced by *Bt 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.

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.

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 non-targets ... 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 non-target 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 non-target insects including predators and parasitoids of lepidopteran insect pests and honeybee."

The Target Pest

European corn borer (ECB) (*Ostrinia nubilalis*) is an important corn insect pest. Physical damage from ECB encompasses a range of effects depending on the number of generations including: a) leaf feeding, b) stalk tunneling, c) leaf sheath and collar feeding, and d) ear damage. Estimated losses from ECB range from 5-10% corn yield annually, due to disruption of nutrient and water translocation, secondary disease infections, stalk lodging, ear drop, and kernel damage.

Appendix C: Genetic Constructs

Two plasmids were used in the biolistic transformation 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. Table 3 outlines the possible array of novel gene and products given the contents of the plasmids used for transformation.

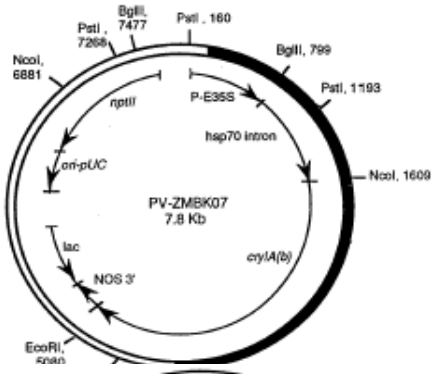


Figure 1. Plasmid map of PV-ZMBK07 showing restriction site locations. Not to scale.

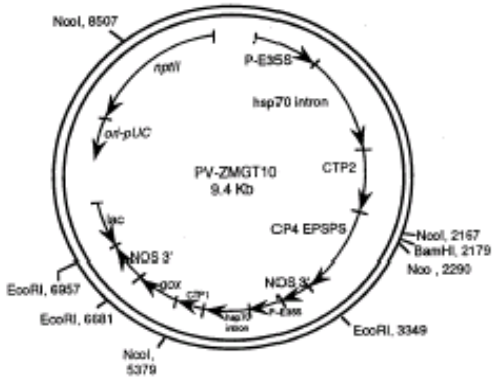


Figure 2. Plasmid map of PV-ZMGT10 with restriction site locations. Not to scale.

Only a portion of the PV-ZMBK07 plasmid vector is present in MON810 and the final MON810 construct does not contain the glyphosate resistance selection genes. Details on how this was determined follow in subsequent sections. "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."

Both plasmids contained the nptII gene encoding for neomycin phosphotransferase II (nptII) under the control of its own bacterial promoter, but the nptII gene was also shown not to be present in MON810. This bacterial gene was used as a selectable marker during plasmid construction.

Table 1: Summary of DNA elements in plasmid PV-ZMBK07

Genetic element	Size Kb	Function
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
hsp 70 intron	0.8	Intron from the maize hsp70 gene (heat shock protein) present to increase the level of gene transcription
cryIA(b)	3.46	The gene encodes the CryIA(b) protein product
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
lacZ	0.24	A partial E. coli lacI coding sequence, the promoter Plac and a partial coding sequence for ?-D-galactosidase or lacZ protein from pUC119
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in E. coli.
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid.

Table 2: Summary of DNA elements in plasmid PV-ZMGT10

Genetic element	Size Kb	Function
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region.
hsp 70 intron	0.8	Intron from the maize hsp70 gene (heat shock protein) present to increase the level of gene transcription
CTP2	0.31	Chloroplast transit peptide (CTP) isolated from Arabidopsis thaliana EPSPS present to direct the CP4 EPSPS protein to the chloroplast, the site of the aromatic amino acid synthesis
CP4 EPSPS	1.4	The gene for CP4 EPSPS, isolated from Agrobacterium sp. strain CP4 which allows for the selection of transformed cells on glyphosate
CTP1	0.26	Chloroplast transit peptide (CTP) isolated from the small subunit gene of ribulose-1,5-biphosphate carboxylase (SSU1A) from Arabidopsis thaliana present to direct the GOX protein to the chloroplast, the site of the aromatic amino acid synthesis
gox	1.3	The gene encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase (GOX) isolated from Achromobacter sp. (new genus Ochrobactrum anthropi) strain LBAA
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
lacZ	0.24	A partial E. coli lacI coding sequence, the promoter Plac and a partial coding sequence for ?-D-galactosidase or lacZ protein from pUC119

ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in E. coli.
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid.

Experiments in corn transformation have demonstrated that the frequency of obtaining transformants containing glyphosate tolerance selection was increased with both plant selectable markers, CP4 EPSPS and GOX. Therefore, both markers were used.

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

Table 3: Possible array of novel gene and products given the contents of the plasmids

novel gene	novel gene product	regulatory sequence	other DNA sequences
PV-ZMBK07			
cryIA(b)	Bt gene	sequence is controlled by E35S promoter (0.6Kb) and a 0.8 Kb intron from the hsp70 gene (heat shock protein) is present to increase the levels of gene transcription. A 0.24 Kb nopaline synthase 3' nontranslated terminator sequence (NOS 3') attached to the cry gene provides the mRNA polyadenylation signals.	
lacZ-alpha	betagalactosidase. A polylinker (region with multiple cloning sites) which allowed the cloning of the desired genes in the plasmid vector	bacteria controlled promoter. Joined at the 3'end of the NOS.	Followed by a 0.7 Kb region of replication for the pUC plasmids (*oripUC*) which allows replication of plasmids in *E. coli.*
NptII (marker for selection during construction of the plasmid) derived from procaryotic transposon Tn5	neomycin phosphotransferase Resistance to aminoglycoside antibiotics (i.e., kanamycin and neomycin)	has its own bacterial promoter	
PV-ZMGT10			

gox gene cloned from *Achromobacter* sp. strain LBAA	glyphosate metabolizing enzyme, glyphosate oxidoreductase (GOX). Degrades glyphosate by conversion to aminomethylphosphonic acid and glyoxylate	joined to CTP1 peptide which targets the gene to the plastids, a chloroplast transit peptide. Derived from a subunit of ribulose -1,5 bisphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana*. Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	

Appendix D: Expression

Samples of field-grown IP corn (MON810) 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 (MON 818 and 819) are not genetically modified, but have "background genetics representative of the test substances." MON 818 is the counterpart for MON810.

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). Overseason 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 4). CP4 EPSPS, GOX and NPTII proteins were not detected. Average protein expression evaluated at six locations was 9.35 µg/g (fwt) in leaves and 0.31 µg/g (fwt) in seeds. Protein expression evaluated at one site was 4.15 µg/g (f.w.) in the whole plant and 0.09 µg/g (fwt) in pollen, as determined from a single sample. Protein expression ranged from 7.93 to 10.34 µg/g (fwt) in leaves, from 0.19 to 0.39 µg/g (fwt) in grain and from 3.65 to 4.65 µg/g (fwt) in the whole plant. Protein expression declined over the growing season as indicated by the Cry1A(b) levels present in leaves assayed over the growing season.

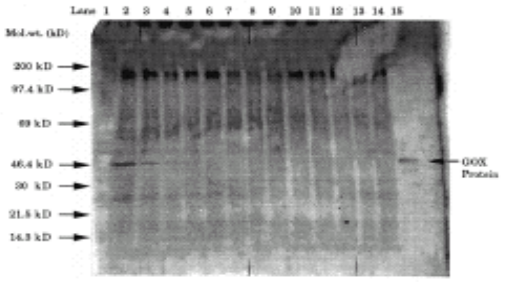
Tissue specificity, as stated by the company, was not expected since the cryIA(b) gene is "under the control of a CaMV promoter. Since this is a constitutive promoter, no specificity of expression to particular tissues is anticipated, although the CaMV promoter may be more or less active in certain cell 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.

Table 4: Summary of levels of protein expression in MON810 tissues[1]

TISSUE	MEAN	STANDARD DEVIATION	RANGE
Btk HD-1			
leaf	9.35	1.03	7.93-10.34
overseason 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, overseason leaf[2], whole plant, grain	nd	-	-
GOX			
leaf, overseason 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.

Western blot analysis of pollen (Figure 3) shows that neither the CP4 EPSPS or the GOX gene were expressed in MON810 (lane 11).



Lane
1) Amersham Rainbow MWM RPN756 batch 43 lot 4
2) 1.0 ng *E.coli* GOX M4-C1 lot 333-73001 spiked into MON 818
3) 0.5 ng *E.coli* GOX M4-C1 lot 333-73001 spiked into MON 818
4) 0.25 ng *E.coli* GOX M4-C1 lot 333-73001 spiked into MON 818
5) 0.125 ng *E.coli* GOX M4-C1 lot 333-73001 spiked into MON 818
6) MON 818 control extract
7) MON 801 extract
8) MON 802 extract
9) MON 808 extract
10) MON 809 extract
11) MON 810 extract
12) MON 813 extract
13) MON 814 extract
14) MON 819 control extract
15) 0.5ng *E.coli* GOX M4-C1 lot 333-73001

Limit of detection (LOD): 0.125 ng GOX/0.1875µg tissue fresh weight
0.67 µg GOX/g tissue fresh weight

Figure 3. Western blot of GOX protein in pollen. Lane 11 is MON810.

For novel food assessments, expression in the consumed portion of the plant, 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 (MON801). The promoter was not active and, therefore, the gene does not express the protein in plant cells.

Appendix E: Transformation Process

Plasmid DNA was introduced into the plant tissue by particle acceleration, or biolistic methods. 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.

Several methods were used to determine the molecular characterization including Southern and Western Blot Analyses. The possible insertion of material based on the plasmid array of genetic material is suggested in tables 1 to 3, describing the DNA components, however, the data indicate this was not the case.

Appendix F: 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.

MON810 is compared against a 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, CP4 EPSPS or GOX proteins.

Insert number

After digestion of extracted DNA with restriction enzyme *Nde*I, which does not cleave within either of the plasmids used to produce MON810, analysis shows that a single band of approximately 5.5 Kb was observed (Figures 3, 4 and 5). 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 and the fragment would contain the inserted DNA and some adjacent genomic DNA. Other bands in the control and sample lanes are considered background (denoted with asterisks).

Insert composition

Digestion with a variety of restriction enzymes (singly and in combination) followed by hybridization with plasmids PV-ZMBK07 and PV-ZMGT10) is used to assess the overall genetic composition of the I-DNA (Figures 3 and 4, Table 5).

Table 5: Restriction enzyme digestion and probe results

Enzyme combinations	Bands (Kb)	Hybridized with PV-ZMBK07	PV-ZMGT10	INTERPRETATION
*Nco*I with *Eco*RI	8.0 2.8	8 8	8	the 2.8 Kb fragment contains the *cryIA(b)* gene which is not present in PV-ZMGT10
*Nco*I with *Bgl*II	5.0 3.0 0.81	8 8 8	8 8	the 3.0 Kb fragment is attributed to the *cryIA(b)* gene. The 5.0 Kb band is weak due to the small amount of complementary DNA in the border fragment
*Pst*I	two 3.1 sized	8	8	
*Pst*I with *Nde*I	3.1 0.8	8 8	8 8	One of the 3.1 fragments was reduced in size. This means that the *Nde*I site closest to the E35S region is nearer the I-DNA than the *Pst*I site.

*Nco*I with *Bam*HI	none	The spiked control gel was a mix of both plasmids.	The spiked control had a 3.1 Kb band, the expected size of the CP4 EPSPS fragment from PV-ZMGT10

Using a number of probes, tests show that the CP4 EPSPS, gox and ori-pUC sequences were not detected in MON810, whereas nptII, E35S, hsp70 and the cryIA(b*)* were present.

Hybridization with the cryIA(b) probe

If the full length cryIA(b) gene occurs in MON810, then a 3.5 Kb fragment should be detected upon digestion with *Nco*I and *Eco*RI, however, a 2.8 Kb fragment (Figure 4, lane 10) is found. This means that one or both of the restriction enzyme sites is missing from the gene.

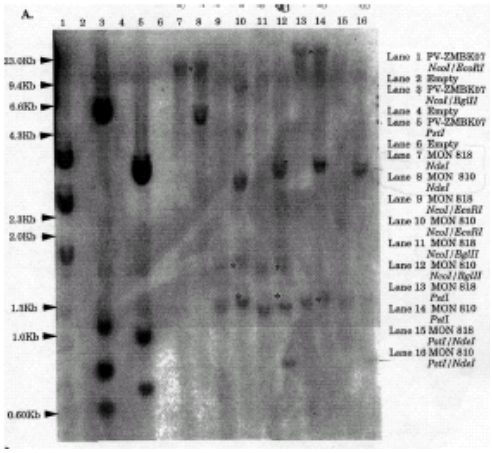


Figure 4. Southern blot DNA digested with a variety of restriction enzyme combinations and probed with plasmidPV-ZMBK07. Lanes 8, 10,12, 14 and 16 are MON810 DNA digested with *Nde*I, *Nco*I/*Eco*RI, E*co*I/*Bgl*II,*Pst*I and *Pst*I/*Nde*I respectively. Asterisks denote background hybridization.

The probe for the hsp70 region indicates that the *Nco*I site is intact, therefore this means that the *Eco*RI site is not present at the 3' end of the gene. Further, the cryIA(b) gene hybridized with the 3.0 Kb fragment in the *Nco*I/*Bgl*II digestion and the 3.1Kb fragments from the *Pst*I digestion, which means these fragments have Cry activity. See figures 4 and 5.

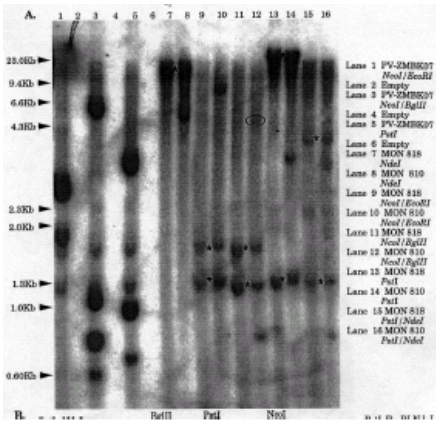


Figure 5. Southern blot of DNA digested with a variety of restriction enzyme combinations and probed with plasmidPV-ZMGT10. Lanes 8, 10,12, 14 and 16 are MON810 DNA digested with *Nde*I, *Nco*I/*Eco*RI, E*co*I/*Bgl*II, *Pst*Iand *Pst*I/*Nde*I respectively. Asterisks denote background hybridization.

Digestion of DNA with *Nco*I/*Eco*RI to release the cryIA(b) gene followed by Southern blot analysis found an approximately 3.1 Kb fragment (Figure 6), which is "sufficient to encode an insecticidally active CryIA(b) protein." While "the positive hybridization control (lane 1 of figure 6) 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 MON810 DNA contains one band of approximately 3.1 Kb."

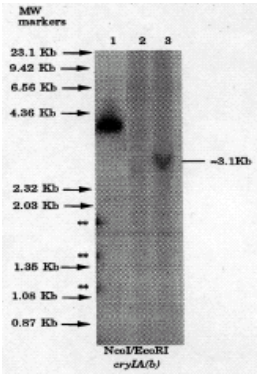


Figure 6. Southern blot of DNA digested with *Nco*I/*Eco*RI and probed with the *cryIA(b)* gene.

Lane 1 plasmidPV-ZMBK07
Lane 2 MON 818 DNA
Lane 3 MON810 DNA

Hybridization with the E35S probe

DNA was digested with *Pst*I alone and in combination with *Nde*I. If the region containing hsp70 and the entire E35S region were present in MON810 then a 1.0 Kb fragment would occur with *Pst*I digestion, but two 3.1 Kb fragments were found. When the *Pst*I was combined with *Nde*I digestion, one of the 3.1Kb fragments was reduced to 0.9 Kb (Figure 7). The E35S probe also hybridized with the 8.0 Kb fragment of the *Nco*I/*Eco*RI digestion and the 5.0 Kb fragment of the *Nco*I/*Bgl*II digestion.

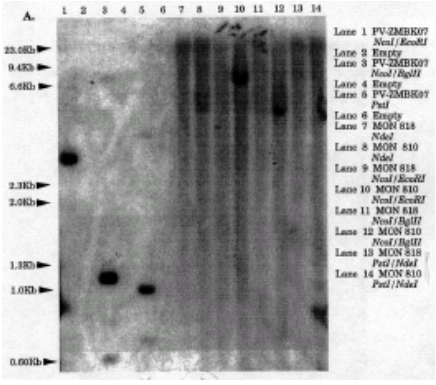


Figure 7. Southern blot of DNA digested with a variety of enzyme combinations and probed with E35S. Lanes 8, 10, 12 and 14 are MON810 digested with *Nde*I, *Nco*I/*Eco*RI, *Nco*I/*Bgl*II and *Pst*I/*Nde*I respectively. Lanes 7, 9, 11 and 13 are for MON 818 with the same enzymes.

Hybridization with the hsp70 probe

If the entire hsp70 intron exists in MON810, digestion with *Nco*I and *Bgl*II would result in a 0.81 Kb fragment. The detection of an 0.8 Kb band means that both the *Nco*I and *Bgl*II sites were present and intact and establishes an intact hsp70. Other evidence to support a complete hsp70 (Figure 8) includes that the probe also hybridizes with the 8.0 Kb fragment in the *Nco*I/*Eco*RI digestion, the 3.1 Kb fragment in the *Pst*I digestion and the 3.1 Kb and 0.8 Kb fragments in the *Pst*I/*Nde*I digestion.

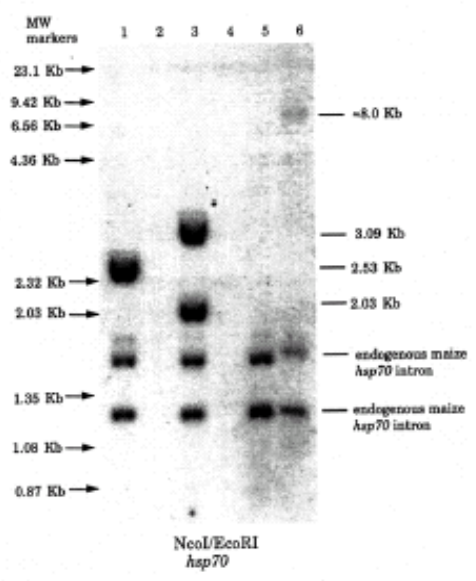
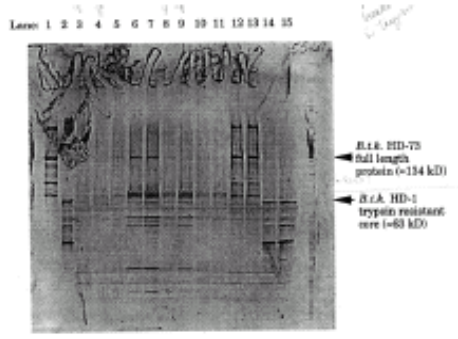


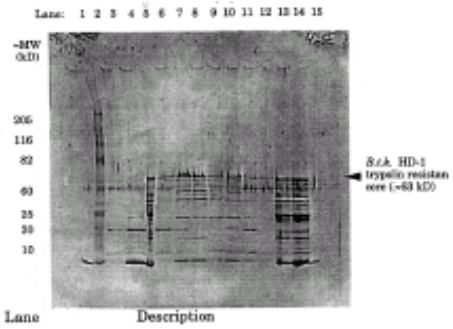
Figure 8. Southern blot of DNA digested with *Nco*I/*Eco*RI and probed with hsp70. Lane 1-MON 818 with PV-ZMBK07Lane 3 -MON 818 with PV-ZMGT10Lane 5 is MON 818 DNA Lane 6 is MON810 DNA. The other lanes are blanks.

To further investigate the composition of the insert, a genomic clone containing the 3' region was isolated and characterized. Sequencing of the clone established that the *Eco*RI site was not present and identified the termination point of the integration event. Sequence analysis shows that the DNA terminates at position 2448 bp and that a maximum open reading frame of 2454 nucleotides is present with the insert beginning at nucleotide 1 of the gene. This frame codes for a protein containing 1-816 of the *Btk* HD-1 protein plus two additional amino acids followed by a stop codon.

Western blots indicate that the trypsin resistant protein of 63Kb is produced by the integrated partial cryIA(b) gene in MON810 (Figures 9 and 10). “Based on the Western blot data and efficacy of maize line MON810, the *cryIA(b)* gene present produces an insecticidal CryIA(b) protein which provides effective, season long control of ECB.”



Lane	Description
1	*E. coli*-produced *B.t.k.* HD-73 full length protein, 20 ng loaded
2	*E. coli*-produced *B.t.k.* HD-1 trypsin-resistant core, 20 ng loaded
3	MON 818 leaf protein extract, 28 µg loaded
4	MON 819 leaf protein extract, 24 µg loaded
5	MON 801 leaf protein extract, 39 µg loaded
6	MON 802 leaf protein extract, 26 µg loaded
7	MON 805 leaf protein extract, 32 µg loaded
8	MON 809 leaf protein extract, 26 µg loaded
9	MON 810 leaf protein extract, 26 µg loaded
10	MON 813 leaf protein extract, 27 µg loaded
11	MON 814 leaf protein extract, 20 µg loaded
12	*E. coli*-produced *B.t.k.* HD-73 full length protein, 20 ng spiked into 19 µg of MON 818 leaf protein extract
13	*E. coli*-produced *B.t.k.* HD-73 full length protein, 20 ng spiked into 16 µg of MON 819 leaf protein extract
14	*E. coli*-produced *B.t.k.* HD-1 trypsin-resistant core, 20 ng, spiked into 19 µg of MON 818 leaf protein extract
15	*E. coli*-produced *B.t.k.* HD-1 trypsin-resistant core, 20 ng, spiked into 16 µg of MON 819 leaf protein extract



Lane	Description
1	Blank lane, 1X SepraSol
2	Color molecular weight markers from Sigma
3	MON 818 leaf protein extract, trypsinized
4	MON 819 leaf protein extract, trypsinized
5	*E. coli*-produced *B.t.k.* HD-1 trypsin-resistant core protein standard, 20 ng
6	MON 801 leaf protein extract, trypsinized
7	MON 802 leaf protein extract, trypsinized
8	MON 805 leaf protein extract, trypsinized
9	MON 809 leaf protein extract, trypsinized
10	MON 810 leaf protein extract, trypsinized
11	MON 813 leaf protein extract, trypsinized
12	MON 814 leaf protein extract, trypsinized
13	*E. coli*-produced *B.t.k.* HD-1 trypsin-resistant core protein standard, 20 ng, spiked into MON 818 extract
14	*E. coli*-produced *B.t.k.* HD-1 trypsin-resistant core protein standard, 20 ng, spiked into MON 819 extract
15	Blank lane, 1X SepraSol •Protein load not determined for the corn extracts •7.5 µl of each corn extract was loaded in 15 µl total volume (lanes 3-6, 8-12) •When spiked with standards (lanes 13-14), 5 µl of the control corn line extracts (MON 818 and MON 819) were loaded in 10 µl total volume

Figure 9 (left). Western blot of *Btk*HD-1 proteins in corn tissue. MON810 is in lane 9.

Figure 10 (right). Western blot of trypsinized BTKHD-1 proteins in corn tissue extracts. MON810 is in lane 9.

CP4 EPSPS probe

Digestion with *Nco*I/*Bam*HI would release any CP4 EPSPS genes present. Southern blots (Figure 11) indicate that MON810 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 12, lane 9).

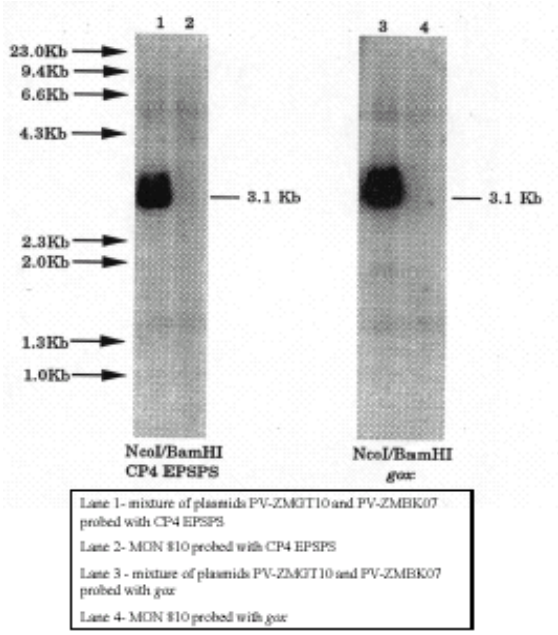


Figure 11. Southern blot of DNA digested with *Nco*I/*Bam*HI and probed with CP4 EPSPS (left) and *gox*genes (right).

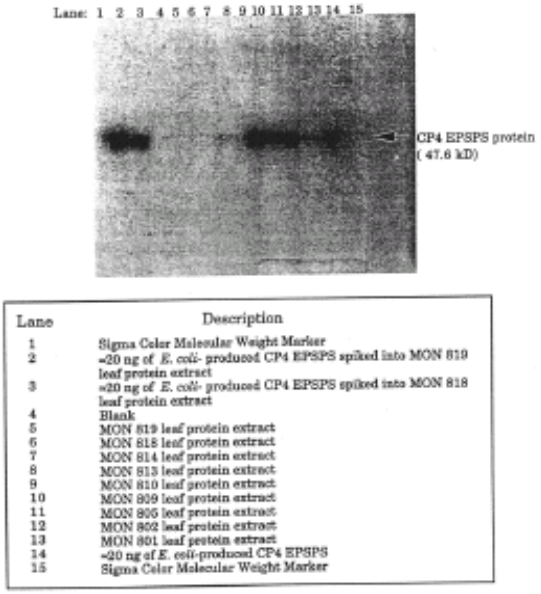
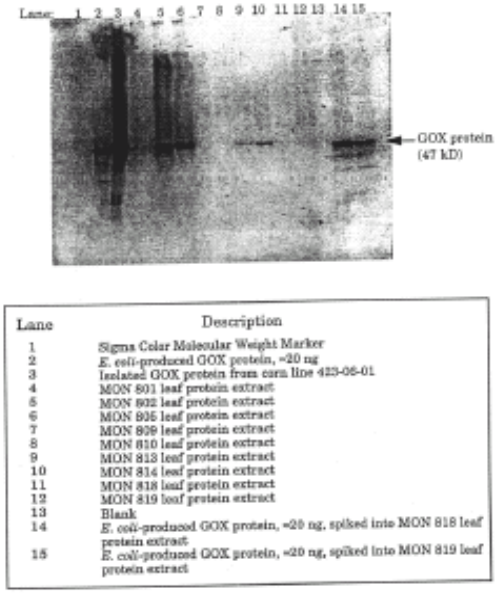


Figure 12. Western blot analysis of CP4 EPSPS protein in corn tissue. MON810 is in lane 9.

Gox gene integrity

Digestion with *Nco*I/*Bam*HI would excise the *gox* gene, if present (*Nco*I to *Nco*I) and would be about 3.1 Kb in size. Southern blot analysis (Figure 11) indicates that MON810 does not contain the *gox* gene. Neither was it detected by ELISA of plant tissues nor by Western blot analysis (Figure 13, lane 8).



Lane	Description
1	Sigma Color Molecular Weight Marker
2	*E. coli*-produced GOX protein, ~20 ng
3	Isolated GOX protein from corn line 423-06-01
4	MON 801 leaf protein extract
5	MON 802 leaf protein extract
6	MON 805 leaf protein extract
7	MON 809 leaf protein extract
8	MON 810 leaf protein extract
9	MON 813 leaf protein extract
10	MON 814 leaf protein extract
11	MON 818 leaf protein extract
12	MON 819 leaf protein extract
13	Blank
14	*E. coli*-produced GOX protein, ~20 ng, spiked into MON 818 leaf protein extract
15	*E. coli*-produced GOX protein, ~20 ng, spiked into MON 819 leaf protein extract

Figure 13. Western blot analysis of GOX protein in corn tissue. MON810 is in lane 8.

Backbone integrity

Backbone (*nptII/ori-pUC) DNA* would be detected following *Nco*I/*Eco*RI digestion with bands at 2.5 Kb and 1.8 Kb when probed with plasmid PV-ZMBK07 and the 1.8 Kb band for both plasmids (the *ori-pUC*). Southern blots (Figure 14) indicate that MON810 contains no backbone sequences. The PV-ZMGT10 plasmid produced two bands at 1.5 Kb and 3.0 Kb, the predicted backbone fragments of the plasmid.

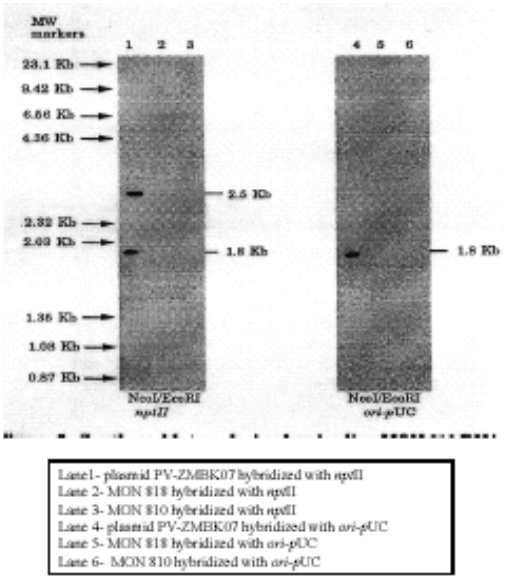


Figure 14. Southern blot analysis of DNA digested with *Nco*I/*Eco*RI and hybridized with backbone sequences, *nptII*(left) and *ori-Puc*(right).

The Southern blot probed with *hsp70* indicates that MON810 contains two endogenous hsp70 bands (1.2 and 1.5 Kb) and an 8.0 Kb band which contains the intron associated with the *cryIA(b)* gene (Figure 8). This demonstrates a single copy of the gene. As DNA probed with nptII/ori-pUC probe produced no bands this demonstrates a lack of backbone sequence.

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 MON810, as shown in the schematic in Figure 15. No DNA from the bacterial vector backbone (e.g., the pUC-origin of replication), the *nptII*, *gox* or CP4EPSPS genes were detected. The submission states that, "MON810 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 MON810.

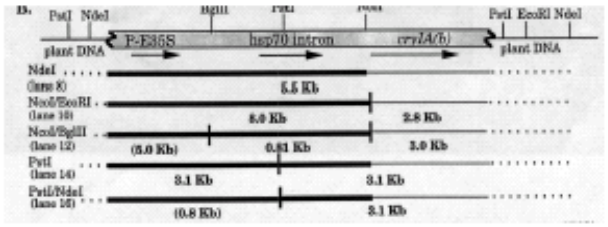


Figure 15. Schematic of inserted DNA from blot analysis. Not to scale.

Plasmid DNA was introduced into the plant tissue by particle acceleration, or biolistic. 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.

Several methods were used to determine the molecular characterization including Southern and Western Blot Analyses. The possible inserted material based on the plasmid array of genetic material is speculated in Table 3, however, the data below indicate this was not the case.

Appendix G: Trait Integrity

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 MON810.

Molecular analysis of MON810 "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. MON810 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 Traits

The full length gene encoding for CryIA(b) protein has been described. While the genes inserted into MON810 have been modified to enhance expression in corn, the amino acid sequence of inserted protein is identical to natural protein derived from Btk. The cryIA(b) gene fragment (Table 6) inserted into the MON810 has been shown to be equivalent to the original bacterium source, as far as activity against insect pests (see below).

Table 6: 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 and in soil

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 protein products, and
- to determine if the expressed *BTK* protein was converted to the expected size of 63 kD trypsin- resistant protein product. Since the CP4 EPSPS AND *gox* genes were not present in MON810, Western blots were not performed for these proteins (Figures 9 and 10).

The company states, "As is commonly observed in Western blot analysis of B.t. proteins, multiple protein products were observed for line MON810 and the other six insect protected corn lines (Figure 9, lanes 5-11). The full length gene was not observed in line MON810, as expected since the full length gene was not incorporated into the

corn genome. .... MON810 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 10).

The protein products in MON810 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 Btk 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 Btk HD-1 CryIA(b) from 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 MON810, 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 Btk HD-1 trypsin resistant core from corn and E. coli are equivalent in molecular weight and immunological specificity, both containing a full length Btk protein band at approximately 134 kD and the same trypsin resistant core of approximately 63 kD. Western blots demonstrated that the Btk HD- 1 core from line 754-10-1 and MON810 were equivalent, therefore it is concluded that the protein produced by the E. coli is an appropriate substitute for the protein in MON810.

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. Testing of the CryIA(b) full length protein against the same sequences indicates it also is safe. Digestive fate shows that the protein is rapidly digested and the commercial microbial product DIPEL contains many fragments as well.

Western blots after treatment with trypsin show equivalent bands and that the 63 kD core is in both samples. MON810 produces a protein product whose trypsin resistant core is equivalent to the trypsin resistant core of the Btk 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 MON810 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 MON810 and its counterpart MON 818.

The Western blot analysis (Figure 10) shows a prominent band at the same molecular weight for MON810 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 20kD 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.”

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

MON810 has been crossed into diverse corn genotypes for several generations and the efficacy of the line has been maintained. The molecular characterization of MON810 was from the third generation of backcrossing and therefore the single insert appears to be stably integrated. Segregation data (Table 7) support a single active insert of the cryIA(b) gene segregating according to Mendelian genetics.

Table 7: Segregation data of MON810 progeny

Generation	Description	Actual	Expected	ChiSq
BC0F1[1]	derived from cross of R0 with an inbred line	44:47	45:5:45:5	0.044*
BC1F1[2]	derived from cross of BC0F1 plants to the same inbred line used to cross the R0 plant	10:4	7:7	1.786*
BC1F2 progeny[3]	derived from cross of individual BC0F2 plants by a non-transgenic tested	69:181:77	81.75:163.5:81.75	4.138#

[1]expressed as number of expressing plants: number of non-expressing plants based on ECB feeding assay
[2]expressed as number of expressing plants: number of non-expressing plants based on cryIA(b) ELISA
[3]expressed as number of ear rows with homozygous number of expressing plants: number of ear rows with segregating plants: number of ear rows with homozygous susceptible plants based on ECB feeding assay
* not significant at p=0.05 (chi square = 3.94, 1df)
# not significant at p=0.05 (chi square = 5.99, 2 df)

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 8). The Chi square tests for the backcross to B73 and Mo17 did not deviate from expectations.

Table 8: Stability of gene transfer based on segregation data for backcross derivatives of MON810 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)

Appendix H: Agronomic Data

Germination Tests and Other Basic Data

Tested at six field locations across the US corn belt, germination of MON810 was high (Table 9). This supports the "conclusion that there are no differences in germination or dormancy between" MON810 and the control plant.

Table 9. Field germination results for MON810 and control

Line	Mean germination	Range
MON810	87.1%	71.1-94.3%
CONTROL	90.6%	78.9-98.3%

Disease and Pest Susceptibilities

MON810 (and its sister MON 809) has been tested in the US in over 60 plantings in at least ten states and Puerto Rico since 1992. Monitoring of disease and insect susceptibility (by comparing general vigour and susceptibility) were performed during the field trials. No differences in agronomic quality, disease or insect susceptibility other than ECB corn borer were detected between the transgenic and non-transgenic plants. Diseases observed in the field included: northern leaf blight (*Exserohilum turcicum*), southern leaf blight (*Bipolaris maydis*), bacterial leaf blight (*Erwinia stewartii*), common corn smut (*Ustilago maydis*), maize stripe virus and common maize rust (*Puccinia sorghi*).

Yield Characteristics

Yield was compared from nine locations in the US and insertion of the *cryIA(b)* gene to MON810 does not negatively affect yield when compared against a non-transgenic hybrid with the same hybrid in which one parent was a backcross derived from MON810 (Table 10).

Table 10: Yield comparison (bushels/acre) of non-transgenic and MON810 versions of the hybrids.

	Yield
control	147.09
MON810	154.90

MON810 has been tested in the US in over 60 plantings in at least ten states and Puerto Rico since 1992. Monitoring of disease and insect susceptibility (by comparing general vigour and susceptibility) were performed during the field trials. No differences in agronomic quality, disease or insect susceptibility other than ECB corn borer were detected between the transgenic and non-transgenic plants. Diseases observed in the field included: northern leaf blight (Exserohilum turcicum), southern leaf blight (Bipolaris maydis), bacterial leaf blight (Erwinia stewartii), common corn smut (Ustilago maydis), maize stripe virus and common maize rust (Puccinia sorghi).

Appendix I: Environmental Interactions

Agronomic characteristics and interaction of the PNT in the environment were collected and are presented in Table 11.

Table 11: Environmental data

Characteristic	Comparative Description		Change
	PNT	Counterpart	
Habit (annual, biennial, perennial)	annual	annual	none
Vegetative vigour (biomass)	73.7 cm[1]	74.5 cm[1]	-1.1%
Overwintering capacity (plant counts)	seeds only	seeds only	none
Flowering period	151.7[2]	146.4[2]	+ 3.6%
Time to maturity	154.1[3]	148.6[3]	+ 3.7%
Seed production	181.4 bu/acre[4]	177.0 bu/acre[4]	+ 2.5%
Dormancy	poor	poor	none
Reproductive characteristics			
- outcross frequency within species	same as counterpart	same as PNT	none
- cross pollination vectors	same as counterpart	same as PNT	none
- fertility- male	yes	yes	none
- fertility- female	yes	yes	none
- self compatibility	yes	yes	none
- asexual	no	no	none
Stress adaptation			
- biotic	ECB resistant	ECB susceptible	PNT is protected
- abiotic	none	none	none
- pesticide	none	none	none
Residual effects	none	none	none
Composition			
- protein	13.1%	6-16.1%	None
- lipid	3.0%	2.9-6.1%	None
- others	82.4000000000006	82.700000000000003	-0.4%
Endogenous toxins (define)	NA	NA	
Non-endogenous toxins (define)	CryIA(b)	none	CryIA(b), ECB resistance
Other observations	NA	NA	

[1]plant height, mean of five experimental hybrids
[2]accumulated temperature (heat units) required to reach pollen shed stage
[3]accumulated temperature (heat units) required to reach silking stage
[4]yield in absence of ECB

Potential for Weediness and Invasiveness

Past experience indicates that corn is not normally a weedy plant based on physiology (the enclosed husk). When harvested corn is transported and kernels are spread along the roadside, "volunteer corn is not found growing in fence rows, ditches and road sides as a weed. ... although corn seed can overwinter into a crop rotation with soybeans, mechanical and chemical measures are utilized for control. ... Corn cannot survive without human assistance and is not capable of surviving as a weed."

Reproductive And Survival Biology

The submission states that phenotype of transformed plants will be very similar to the original phenotype and its ability to survive as a weed will not change. Observations from field trials over the 1993-1995 seasons "demonstrate that the mode and rate of reproduction of insect-protected corn line MON810 is typical of other corn." MON810 exhibits the same separate staminate (tassel) and pistillate (silk) features and pollen is produced entirely in the staminate inflorescences with anthesis (pollen shed) synchronous with silk emergence. No differences in seed or plant maturity were observed, though in some trials, non-insect-protected plants matured more rapidly due to ECB damage causing premature senescence. Yield and seed germination (see above) were similar to the control. "There is no change in reproductive or survival biology associated with the insect-protected phenotype."

Adaptation to Stress

The company offers the following on the subject, "insect-protected corn line MON810 has been grown in diverse environments in the North American corn belt and in other countries. The adaptation of line MON810 to stress has not been altered as a result of the genetic modification, except for protection afforded against feeding damage by ECB. Populations of ECB are highly variable, season to season, and are not a limiting factor in corn production in Canada. The disease and pest susceptibilities of MON810 are otherwise unchanged. More recently, academic research has indicated a potential reduction in the occurrence of stalk rots associated with ECB damage (Fusarium sp.) to the corn ear and associated production of harmful mycotoxins."

Outcrossing with Wild *Zea* Species

Corn freely crosses with teosinte in Mexico and Guatemala where teosinte exists primarily as a weed around cultivated corn fields. In one study in Mexico, the frequency of hybrids was around 2-5% of the teosinte population. This is a "significant gene exchange between a weedy plant (i.e., teosinte) and a cultivated relative (i.e., corn)." The F1 hybrid is robust and fertile and can be backcrossed with corn.

The range of teosinte is the seasonally dry, subtropical zone along the western escarpment of Mexico and Guatemala and the central plateau of Mexico. Except for special plantings it is not grown in the US and there are no reports of it growing as a weed along the margins of corn plantings in the US.

Another wild relative is Tripsacum. Wild hybrids of Tripsacum with corn have not been observed but crossing occurs only under special circumstances and the offspring are often sterile. Tripsacum spp. (16 in number) are native to south and central America.

The submission states that the outcrossing of transformed plants will be the same as for nontransformed plants.

Outcrossing with Cultivated *Zea* Varieties

Corn is wind pollinated, so distances that viable pollen can travel depend on wind level and patterns, humidity and temperature. Under favourable conditions, corn pollen has been found to travel up to 3.2 kilometers. Most corns (flint, dent, sweet, pop) will interpollinate (exceptions include some popcorns). “Corn pollen is very promiscuous” and each corn plant can produce more than 10 million pollen grains.

“Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time. ... the chance that a weedy type of corn will result from outcrossing with cultivated corn seems extremely remote. Free flow of genes would occur similarly to what occurs naturally. The production of B.t.k. CryIA(b) protein in resulting seed would not be an issue due to the safety demonstrated for insect protected corn.”

Recommended certification standard distances (at the time of the submission) between different corn genotypes for commercial hybrid seed production are that the seed parent should be no less than 200 m from other corn of a similar type. The distance can be modified based on the field size, number of border rows and maturity dates for flowering. The submission noted that if the hybrid seed being produced is of a different colour or texture from neighbouring contaminating fields, then the distances and number of border rows should be increased.

Altered Plant Pest Potential

Agronomic characteristics of the modified corn hybrids were shown to be within the range of values displayed by currently commercialized corn hybrids, and indicate that the growing habit of corn was not inadvertently altered. Field observations did not indicate modifications of disease and pest susceptibilities, other than to ECB.

Appendix J: Trait Specificity

Nontoxic Claims

The submission summarizes potential nontarget impacts with: “the naturally occurring B.t. proteins have been demonstrated to be virtually non-toxic to fish, avian species, mammals and other nontargets. Since the naturally occurring B.t.k proteins have been demonstrated to be virtually non-toxic to fish, avian species , nontarget insects, mammals and other nontarget species, no adverse effects are expected to wildlife from the commercialization of these plants.”

“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).” Application of conventional chemical insecticides often affect nontarget species.

Tests cited from the literature include tests for commercially available microbial pesticide products such as DIPEL®, the subject of numerous safety assessments for pesticidal registrations, note these are “widely recognized as nontoxic for mammals, birds and fish as well as beneficial nontarget insects including predators and parasitoids of lepidopteran insect pest and honeybee.”

Using the concept of equivalency, they state that the safety data submitted for microbial products containing the protein “can be applied to the safety assessment of the protein expressed in MON810 and corn inbreds and hybrids derived from this line.”

Tests were done using the trypsin-resistant core, E.coli produced protein described elsewhere in the document, shown to be equivalent to the plant expressed protein.

Tests done using trypsin-resistant core protein (not pollen, plant tissue or extracts)

Honey bee (Aphis mellifera L.) larvae and adults
Honey bees may feed on the pollen of corn pollen. The stability of the trypsin core protein in sucrose and honey solutions under non-refrigerated conditions was confirmed and a maximum hazard dose calculated, a concentration of the protein greater than ten times the estimated level required for 50% mortality (LC50) of several target pest Lepidoptera. The LC50 in honey bee larvae and adult honey bee was greater than 20 ppm, the highest dose administered, therefore the No Observed Effects Level (NOEL) was set at 20 ppm.

Green lacewing larvae (Chrysopa carnea)
Lacewings, beneficial predatory insects, are found in corn fields. There was no evidence of adverse effects when larvae were fed moth eggs coated with a nominal concentration of 16.7 ppm of the CryIA(b) protein for seven days. Therefore, the LC50 was set at 16.7 ppm, the highest dose tested.

Parasitic hymenoptera
The test organism (Brachymeria intermedia), a beneficial parasite of the housefly (Musca domestica), was exposed to the protein in a concentration of 20 ppm in a honey/water solution for thirty days. The wasps exhibited no signs of toxicity or treatment related mortality and the LC50 and NOEL were set at 20 ppm, the highest dose administered.

Lady bird beetles (Hippodamia convergens)
Lady bird beetles, common beneficial predaceous insects that feed on aphids and other plant insects, are commonly found on weeds and crops. Fed the same test solution as the wasps for nine days, they also exhibited no mortality or signs of toxicity associated with the treatment, therefore the LC50 and the NOEL were set at 20 ppm, the highest dose.

Collembola
Feeding tests with insecticidal proteins, including the one inserted into MON810, were done on two species of Collembola (springtails- Folsomia candida, Xenylla grisea) nontarget soil invertebrates that could come into contact with the B.t. proteins from crop residue during decomposition in the soil. Studies indicate that the biological activity of CryIA(b) protein from corn dissipates rapidly in the soil, with 50 and 90% of the activity gone in 2 and 15 days respectively. The purpose of these toxicological tests was to assess potential ecological risks to beneficial nontarget organisms. The proteins (at 200 ppm) and positive control (chemical insecticide Chloropyrifos as Lorsban 4E) were incorporated into brewer's yeast and freeze dried to prepare a diet for the insects and insects were exposed in Petri dish microcosms for 21 days. The conclusion is that Bt proteins pose no identifiable toxicological risk to soil inhabiting Collembola species (Table 12).

Table 12: Survival and reproduction of Collembola species exposed to Bt proteins and controls (means ± SD)

Treatment	*F. candida* (adults)	*F. candida* (progeny per adult)	*X. grisea* (adults + progeny)
Bt protein cryIA(b)	9.8 ± 0.4	15.1 ± 3.0	98.8 ± 50.7
Control for Bt	9.6 ± 0.6	13.7 ± 2.3	120.7 ± 32.1
Chloropyrifos- 200 ppm	0	0	16.8 ± 12.4
Chloropyrifos - 20 ppm	0.25 ± 0.5	0	15.3 ± 10.2
Chloropyrifos - 2.0 ppm	9.8 ± 0.5	13.3 ± 3.1	56.0 ± 44.6
Chloropyrifos - 0.2	9.0 ± 0.8	14.5 ± 1.3	28.8 ± 21.6
Chloropyrifos - 0.0	9.3 ± 1.2	12.5 ± 1.3	40.3 ± 20.7

Earthworm
Fourteen day earthworm (Eisenia fetida) tests found that the LC50 of the trypsinized CryIA(b) is greater than 200 mg/kg of dry soil, with the NOEC set at 200 mg/kg dry soil

(200 ppm). Observations made on the earthworms include burrowing behaviour and body weights. The positive control was chloroacetamide at concentrations of 14, 30 and 60 mg a.i./kg dry soil. Average mortality for the control (15%) and for the 200 mg/kg of CryIA(b) protein (25%) were not significantly different. Average body weight (mg) for the controls changed by about -5 with a 57.5 mg increase for the earthworms exposed to the cryIA(b) protein. “Change in body weight was variable between the individual replicates. However, there did not appear to be any treatment-related effects upon body weight.” For the chloroacetamide, mortality was 100 % for the 30 and 60 mg/kg trials and 30 % for 15 mg/kg addition to the soil. The average reduction in body weight for the 15 mg/kg group was 37.5 mg.

Fate of the CryIA (B) Protein

Corn residue, containing low levels of the CryIA(b) protein may be tilled into the soil or stay on the soil surface (zero/conservation tillage). Environmental fate was determined by measuring the rate at which the CryIA(b) protein dissipated when added to soil as a purified protein and as a component of corn tissue. The levels for tests incorporated into soil were three times higher than the maximum concentration expected under field conditions.

The dissipation rate (DT50) of Cry IA(b) protein in 3 systems: 1) corn without contact with soil; 2) corn mixed with soil and 3) purified protein mixed in soil were: 1) 25.6 days, 2) 1.6 days and 3) 8.3 days. This rate of dissipation is comparable to that observed with microbial Btk products. This indicates that it will dissipate readily on the surface (no till condition) and when tilled into soil and should have no deleterious effects on soil microflora and fauna.

Potential Effects on Biodiversity

As previously stated, the B.t.k. protein is indigenous to the environment and is not known to be toxic to mammals, fish, birds and other nontarget species, therefore, no adverse effects are expected to wildlife. “No endangered or threatened lepidopteran insects as listed on 50CFR 17.11 or 17.12, feed on corn plants.” Table 13 summarizes interactions and effects in managed and natural ecosystems.

Table 13: Effects of PNT on ecosystem parameters

	Natural ecosystem				Managed ecosystem			
Effects of release	Degree of change	Geographic scope	Duration	Relative impact	Degree of change	Geographic scope	Duration	Relative impact
Biodiversity: plant populations	0	national	years	0	0	local	months	0
Animal populations	0	"	"	0	0	"	"	0
Microbe populations	0	"	"	0	0	"	"	0
Substance presence/ persistence	0	"	"	0	0	"	"	0
Sustainability	0	"	"	0	+	"	"	+
Agronomic/ silvicultural practices	0	"	"	0	+	"	"	+
Resource conservation	0	"	"	0	0	"	"	0
Other concerns (e.g., occupational health and safety)	0	"	"	0	+	"	"	+
Overall environmental quality changes	0	local		0	+	"	"	+

Appendix K: Cultivation

Proposed Production Area

MON810 will be available for use in all corn growing areas, but is not expected to alter the normal geographical regions for corn production or result in a significant increase in the cultivated area planted to corn, since ECB infestation is not a limiting production factor.

Altered Cultivation Practices

“Cultivation practices, harvest and post-harvest protocols will not vary from those used traditionally for the cultivation of corn crops and distribution of corn products in Canada.” The following benefits are listed:

- more reliable and economical control method for ECB
- equal benefit to large and small growers since no additional labour, planning or machinery is required
- reduction in the use of insecticides to control ECB
- reduction in manufacturing, shipment and storage of chemical insecticides
- reduction in worker exposure to insecticides
- good fit with IPM and sustainable agricultural systems
- insect control without harming nontarget species and beneficial insects
- potential reduction in the occurrence of stalk rots associated with ECB damage to corn ear and to mycotoxin production

Appendix L: Toxicity and Allergenicity

The MON810 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 2632 amino acid sequences of 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 16.

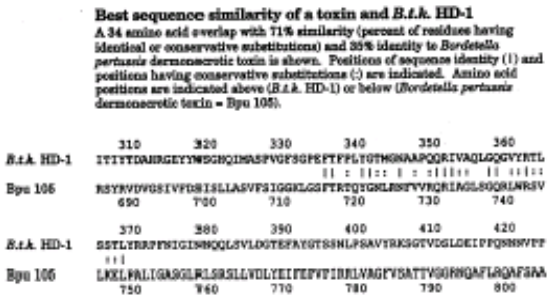


Figure 16. Best sequence homology of a toxin and *Btk*HD-1 protein.

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 14) 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.

Table 14: 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)	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]

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 0.19-0.39µg fresh weight of corn seed.

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 found in Figure 17. The results establish that B.t.k. HD-1 protein shares no significant similarity with known allergen or gliadin proteins.

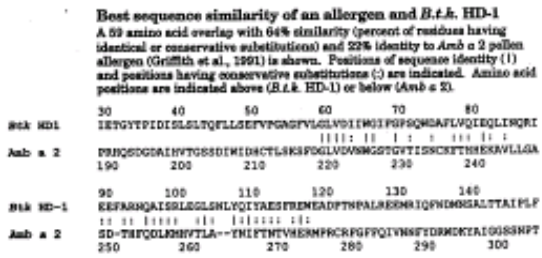


Figure 17. Best sequence similarity of an allergen and *BTK*HD-1 protein

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.

Appendix M: Feeding Study

Bobwhite Quail (*Colinus Virginianus*) Feeding Study

The purpose of this study (note this study was done with another line of IP corn, MON 801) is to assess the wholesomeness of insect protected corn meal fed to quail. The birds were fed up to 10% w/w (100,000 ppm) raw corn seed meal which is equivalent to 138 seeds/kg body weight/bird/day. No mortality was observed and no differences were found in body weight, food consumption (Table 15), appearance or behaviour between the control and the IP corn during the 5 day test.

The NOEL was considered to be greater than 10% w/w and the IP corn seed was considered comparable in wholesomeness to the parent control. “No additional feeding studies were conducted with MON810 corn seed or meal.”

Table 15: Bobwhite quail feeding study (5 day)

Group	Test (ppm)	Body weight(g)		Food consumption (g/bird/day)	
		pre	post	day 0-1	day 4-5
Basal diet	0	20 ± 3	28 ± 4	9	7
	0	19 ± 2	26 ± 3	6	5
	0	21 ± 3	30 ± 6	9	8
Parent	50000	20 ± 3	30 ± 4	7	8
	100000	20 ± 3	28 ± 3	7	10
IP corn	50000	20 ± 3	31 ± 3	9	8
	100000	20 ± 3	29 ± 5	8	9

Appendix N: Compositional Data

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 MON810 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 16

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

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

Table 16: Comparison of compositional analysis for MON810 corn grain with control (MON 818) and literature values

Component	MON810[1] mean (range)[2]	MON 818 mean (range)[2]	Literature value[4] mean (range) [MON 800/801 range]
PROXIMATE ANALYSIS			
Protein[3]	13.1 (12.7-13.6)	12.8 (11.7-13.6)	9.5 (6.0-12.0) 12.3 (9.7- 16.1) [11.2-13.6]
Fat	3.0 (2.6-3.3)	2.9 (2.6-3.2)	4.3 (3.1-5.7), 4.6 (2.9-6.1) [3.8-4.2]
Ash[3]	1.6 (1.5-1.7)	1.5 (1.5-1.6)	1.4 (1.1-3.9) [1.5-1.8]
Carbohydrate[3]	82.4 (81.8-82.9)	82.7 (81.7-83.8)	not reported [80.8-83.0]
Calories/100g	408.4 (407.0-410.1)	408.5 (406.0-410.1)	not reported [412.6-415.7]
Moisture %	12.4 (11.0-14.4)	12.0 (10.6-14.2)	16.0 (7-23) [13.0-15.8]

AMINO ACID COMPOSITION - NUTRITIONALLY ESSENTIAL[5]			
Methionine	1.7 (1.6-1.9)	1.7 (1.6-1.7)	1.0-2.1 [2.0-2.6]
Cystine	2.0* (1.9-2.1)	1.9 (1.8-2.0)	1.2-1.6 [1.9-2.3]
Lysine	2.8 (2.5-2.9)	2.8 (2.7-2.9)	2.0-3.8 [2.6-3.4]
Tryptophan	0.6* (0.5-0.7)	0.6 (0.4-0.6)	0.5-1.2 [0.5-0.6]
Threonine	3.9 (3.7-4.4)	3.8 (3.7-3.9)	2.9-3.9 [3.9-4.2]
Isoleucine	3.7 (3.3-4.1)	3.8 (3.6-4.0)	2.6-4.0 [3.5-3.8]
Histidine	3.1* (2.9-3.3)	2.9 (2.8-3.0)	2.0-2.8 [2.8-3.3]
Valine	4.5 (4.1-4.9)	4.6 (4.3-4.8)	2.1-5.2 [4.2-4.8]
Leucine	15.0 (14.1-16.7)	14.5 (13.8-15.0)	7.8-15.2 [13.6-14.5]
Arginine	4.5 (4.2-4.7)	4.5 (4.2-4.7)	2.9-5.9 [4.1-5.0]
Phenyalanine	5.6* (5.2-5.6)	5.4 (5.2-5.6)	2.9-5.7 [5.2-5.6]
Glycine	3.7 (3.4-4.0)	3.7 (3.5-3.8)	2.6-4.7 [3.4-4.2]
AMINO ACIDS - NONESSENTIAL[5]			
Alanine	8.2* (7.8-8.9)	7.8 (7.5-8.0)	6.4-8.0 [7.8-8.2]
Aspartic acid	7.1 (6.4-8.2)	6.6 (6.3-6.8)	5.8-7.2 [6.7-7.3]
Glutamic acid	21.9 (20.4-24.4)	21.1 (201.-21.6)	12.4-19.6 [19.9-21.4]
Proline	9.9* (9.7-10.5)	9.6 (9.4-9.8)	6.6-10.3 [9.0-9.4]
Serine	5.5* (5.3-5.9)	5.2 (5.1-5.4)	4.2-5.5 [5.5-6.1]
Tyrosine	4.4* (4.1-4.8)	4.0 (3.9-4.1)	2.9-4.7 [3.8-4.3]
FATTY ACIDS[6]			
Palmitic (16:0)	10.5 (10.2-11.1)	10.5 (10.2-10.7)	7-19 [10.2-10.9]
Stearic (18:0)	1.9 (1.7-2.1)	1.8 (1.8-1.9)	1-3 [1.6-3.1]
Oleic (18:1)	23.2 (21.5-25.4)	22.8 (21.6-23.9)	20-46 [21.2-25.9]
Linoleic (18:2)	62.6 (59.5-64.7)	63.0 (61.8-64.6)	35-70 [58.9-65.0]
Linolenic (18:3)	0.8 (0.7-0.9)	0.9 (0.8-0.9)	0.8-2 [0.9-1.1]
CARBOHYDRATES AND FIBER[7]			
Starch %	67.6 (65.3-69.7)	66.9 (64.6-69.0)	64-78.0 [63.7-71.5]
Crude fiber %	2.6* (2.5-2.8)	2.4 (2.3-2.5)	2.0-5.5 [1.98-2.61]
Sugars[8] – fructose – glucose – sucrose	 0.32 (0.23-0.35) 0.44 (0.34-0.47) 0.93 (0.79-1.12)	 0.27 (0.22-0.40) 0.93 (0.79-1.12) 0.93 (0.68-1.11)	 [0.47-0.96] [0.47-1.03] [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]
INORGANIC COMPONENTS[7]			
calcium %	0.0036* (0.0033-0.0039)	0.0033 (0.0029-0.0037)	0.01-0.1 [0.003-0.004]
phosphorus %	0.358 (0.334-0.377)	0.348 (0.327-0.363)	0.26-0.75 [0.311-0.368]

[1]values with * are statistically different from MON 818
[2]values reported are means of six samples from six sites. Ranges are the highest and lowest values across those sites.
[3]percent dry weight of samples
[4]where there are more than one value, this indicates more than one published source
[5]values for amino acids reported as percent of total protein
[6]values for fatty acids are % total lipid. Other fatty acids were below the limit of detection of the assay
[7]values on a dry weight basis
[8]sugars measured as g/100g. Galactose, lactose and maltose were also measured, but values were below the limit of detection.

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.

MON810 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 MON810 (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 MON810 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 one and is therefore not considered an important component of the corn grain. MON810 values for the alpha and gamma tocopherols were statistically similar to the control but different for the beta tocopherol.

For the minerals calcium and phosphorus, calcium levels in MON810 were statistically higher than for MON 818, but within ranges reported for tests with MON 800/801. No statistical differences were found for phosphorus.

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.”

Nutritional analysis conclusions, “nutritional composition ... falls within the ranges of each nutrient measures for nonmodified 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, MON810 may be considered to be substantially equivalent to regular corn.”