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Application to Food Standards Australia New Zealand for the inclusion of Lepidopteran-protected maize MON 89034 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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GM CHECKLIST

	Data Provided	Part No.	Data Not Provided	Omission Explained
Executive Summary				
Separately bound document	☒		☐	☐
Part 1: General Information				
1.1 Applicant				
(a) Company / Organisation Name	☒	1.1	☐	☐
(b) Address	☒	1.1	☐	☐
(c) Contact	☒	1.1	☐	☐
(d) Nature of business	☒	1.1	☐	☐
(e) Sole or joint application	☒	1.2	☐	☐
(f) Co-applicants	☐	1.2	☐	☒
Part 2: Specific Data Requirements				
2.1 General Details				
(a) Description of GM organism	☒	2.1.a.	☐	☐
(b) Name / Number of new line / strain	☒	2.1.a.	☐	☐
(c) Marketing name	☒	2.1.c.	☐	☐
(d) Product list	☒	2.1.d.	☐	☐
2.2 History of Use				
(a) Donor	☒	2.2.a.	☐	☐
(b) Host	☒	2.2.b.	☐	☐
2.3 Nature of Genetic Modification				
(a) Method used	☒	2.3.a.	☐	☐
(b) Bacteria used	☒	2.3.a.	☐	☐
(c) Gene Construct and transformation event	☒	2.3.b.	☐	☐
(d) Molecular characterisation	☒	2.3.c.	☐	☐
(e) Derivation of line or strain	☒	2.3.d.	☐	☐
(f) Evidence of stability	☒	2.3.d.	☐	☐
2.4 Antibiotic Resistance Genes				
(a) Clinical / veterinary importance	☐	NA	☐	☒
(b) Viability	☐	NA	☐	☒
(c) Presence in food	☐	NA	☐	☒
2.5 Characterisation of Novel Protein				
(a) Description	☒	2.5.a.	☐	☐
(b) Site of expression	☒	2.5.b.	☐	☐
(c) Non-expression	☐	NA	☒	☐
(d) History of human consumption	☒	2.5.c.	☐	☐
(e) Oral toxicological studies	☒	2.5.d.	☐	☐
(f) Amino acid sequence	☒	2.5.e.	☐	☐
(g) Known allergenicity of source	☒	2.5.f.	☐	☐
(h) Unknown allergenicity information	☒	2.5.g.	☐	☐

GM CHECKLIST (continued)

	Data Provided	Part No.	Data Not Provided	Omission Explained
2.6 Characterisation of Other Novel Substances				
(a) Identification	☐	NA	☒	☐
(b) Toxicity	☐	NA	☒	☐
2.7 Comparative Analyses				
(a) Key nutrients etc.	☒	2.6.a.	☐	☐
(b) Other constituents	☒	2.6.b.	☐	☐
(c) Allergenic proteins	☐	NA	☒	☐
2.8 Nutritional Impact				
(a) Animal feeding studies	☐	2.7	☐	☒
(b) Nutritional changes	☐	2.7	☐	☒
2.9 Other Technical Information				
(a) Detection methodology	☒	2.8.a.	☐	☐
(b) Market penetration	☒	2.8.b.	☐	☐
Part 3: Regulatory / Legislative Implications				
3.1 Other approvals				
(a) Relevant overseas approvals	☒	3.1.a.	☐	☐
(b) Approval refusal	☒	3.1.b.	☐	☐
Part 4: Statutory Declaration	☒		☐	☐

PART 1 GENERAL INFORMATION

1.1 Applicant Details

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Nature of Your Business:	Technology Provider to the Agricultural and Food Industries.

1.2 Nature of Application

This application is submitted to Food Standards Australia New Zealand by Monsanto Australia Limited and is not made on behalf of any other party.

The purpose of this submission is to make an application to vary **Standard 1.5.2** – *Food Produced Using Gene Technology* to seek the addition of Lepidopteran-protected maize MON 89034 and products containing Lepidopteran-protected maize MON 89034 (hereafter referred to as MON 89034) to the Table to Clause 2 (see below).

Column 1 **Food derived from gene technology**	**Column 2** **Special requirements**
Food derived from Lepidopteran-protected maize MON 89034	None

PART 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

2.1 Background Details

a) Description of the nature and purpose of the introduced trait

Maize (or corn[1]) yields and grain quality are significantly impacted by insect pests resulting in crop losses (James, 2003). In 1997, Monsanto commercialised the biotechnology-derived product in the United States, YieldGard® Corn Borer maize (hereafter referred to as MON 810) that contains the *cry1Ab* gene from *Bacillus thuringiensis* (Bt) which encodes the Cry1Ab protein that provides effective protection against damage caused by lepidopteran insect pests, especially the European corn borer (ECB, *Ostrinia nubilalis*) and the corn earworm (CEW, *Helicoverpa zea*). At that time in the U.S., the combination of crop losses and management costs resulting from lepidopteran pests were estimated to be over $US1 billion per year (Mason et al., 1996). The introduction of MON 810 and other Bt maize products provided maize growers with a more effective solution for the control of lepidopteran larval pests such as European corn borer and corn earworm. In addition to the benefits of MON 810 as an effective pest management tool, this product also resulted in lower levels of harmful mycotoxins in Bt maize, which has resulted in improved food and feed safety of maize by reducing insect damage and subsequent fungal infections that produce mycotoxins. This reduction has been consistently demonstrated in countries around the world where corn borers are the predominant insect pests (Clements et al., 2003; Dowd, 2000 and 2001; Hammond et al., 2002 and 2004; de la Campa et al., 2005; Bakan et al., 2002; Magg et al., 2002; Munkvold et al., 1999; Munkvold, 2003; Papst et al., 2005; Pietri and Piva, 2000; Wu, 2006). Furthermore, the use of MON 810 and other Bt maize products has reduced the use of chemical insecticides (Carpenter et al., 2004). Within ten years of the first Bt maize product introduction, the safe and effective use of Bt maize has been adopted globally on over 17 million hectares (James, 2005) to control several primary insect pests of maize in twelve countries.

Recently, Monsanto has developed MON 89034 as a second generation product to provide enhanced benefits for the control of lepidopteran pests of maize. MON 89034 was developed using a single transformation vector containing both the *cry1A.105* and *cry2Ab2* genes. This approach, known as vector stacking, increases the efficiency of breeding multiple traits into new maize hybrids, thereby providing growers an earlier access to improved germplasm containing these traits rather than through conventional inbred stacking.

The benefits of MON 89034 provides four key advantages, which are more specifically described below:

1) *Extended spectrum*: A major benefit of MON 89034 is the protection of maize plants from feeding damage caused by lepidopteran insect pest larvae. MON 89034 provides outstanding control of *Ostrinia* species such as

[1] Please note that maize and corn are used interchangeably throughout this document.
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European corn borer and Asian corn borer (ACB), and *Diatraea* species such as southwestern corn borer (SWCB) and sugarcane borer (SCB). Control of these insects provided by MON 89034 is comparable to MON 810. MON 89034 also provides a high level of control of fall armyworm (FAW) throughout the season, whereas MON 810 principally controls damage caused by FAW larvae during vegetative growth. In addition, MON 89034 provides significant protection from damage caused by corn earworm. Introduction of MON 89034 will offer farmers a safe and effective alternative to the use of chemical insecticides for the control of the lepidopteran pests.

2) *Improved IRM:* MON 89034 produces two different Bt proteins, Cry2Ab2 and Cry1A.105, which are both highly efficacious against a variety of lepidopteran pests. The mechanism of insecticidal activity or mode of action of Bt proteins consists of a number of steps, e.g., proteolysis, binding, and pore formation (English and Slatin, 1992). There are important differences in each step of the mechanism of activity that influence the interactions of these proteins with susceptible organisms without qualitatively influencing their host range. Several lines of evidence establish that Cry1A.105 and Cry2Ab2 have important differences in their mode of action, particularly in the way in which they bind to the lepidopteran midgut. These proteins have different primary structures, share only ~14% of amino acid sequence identity, and bind to distinct proteins in the midgut of target species at different rates with different affinities. Therefore, the probability of cross-resistance between these proteins is very low. Furthermore, *in vitro* and *in planta* studies of the Cry1A.105 and Cry2Ab proteins demonstrate that both proteins are highly active against the primary lepidopteran pests of maize: ECB, SWCB, CEW, and FAW. In view of the dual effective dose and the distinct mode of action of the two proteins in MON 89034, the likelihood of resistance evolution is significantly reduced compared to single protein products. Therefore, MON 89034 is expected to be sustainable using a reduced structured refuge.

3) *Mycotoxin reduction:* Lepidopteran pests such as ECB, CEW and FAW cause substantial damage to stalks, ears, and leaves of developing maize plants resulting in reduced yields. MON 89034 has been shown to provide protection against a wider variety of lepidopteran pests than MON 810 and in addition to greater yield protection, this is expected to result in a broader reduction for potential mycotoxin contamination in diverse environments. Maize ears that are protected from feeding damage caused by ECB, CEW, and FAW would have fewer ports of entry for invasion by fungi that produce mycotoxins, which will make the grain safe to consume. As regulations are implemented globally that limit mycotoxin levels in food and feed, the economic impacts of mycotoxin contamination in grain will become increasingly important. With the expanded spectrum of MON 89034 compared to MON 810, it is expected that there will be a greater benefit to food and feed consumed globally.

4) *Efficient Trait Integration:* The rapid development of elite maize hybrids containing the *cry1A.105* and *cry2Ab2* genes is made possible by vector stack technology, i.e., the incorporation of multiple genes into a single transformation event. This approach increases the efficiency of introduction

of both proteins into new maize germplasm by conventional breeding, thereby providing growers early access to a variety of elite maize germplasm containing both insecticidal proteins. Maize hybrids containing multiple Bt proteins conferring insect protection traits have been developed previously using traditional breeding techniques, i.e., two inbreds containing individual traits were crossed to produce the combined trait product. However, such breeding programs are generally costly because of duplicated work to introgress two transformation events into new germplasms. The MON 89034 technology can reduce the time and cost factors in breeding programs to introgress only a single transformation event.

b) Mode of action of the introduced trait

The Cry1A.105 and Cry2Ab2 proteins are produced by the lepidopteran-protected maize MON 89034. The general mechanism of insecticidal activity of Cry proteins is well understood (Gill et al., 1992; Schnepf et al., 1998; Zhuang and Gill, 2003). It has been established that Cry proteins have a narrow range of insecticidal activity against one or, less commonly, two orders of insects (Crickmore et al., 1998). Generally, Cry proteins have a defined spectrum of insecticidal activity within a particular insect order (De Maagd et al., 2001). This high degree of specificity is governed by four levels of selectivity which collectively lead to intoxication (Federici, 2002). The levels of selectivity include: 1) the route by which the insect is exposed to Cry proteins (i.e., ingestion of plant tissues), 2) protein toxin activation by specific proteolytic enzymes (determined by differences in gut physiology between insects), 3) toxin binding to an available specific midgut receptor, and 4) changes in the protein configuration. The reconfigured protein is then able to enter the midgut membrane and form channels. This affects larval ability to feed and develop, and eventually leads to death.

Cry proteins are comprised of several functional domains that have highly conserved regions between the classes. For example, the amino acid sequence of Cry1A proteins is highly conserved in domains I, II and III. These functional domains have been shown to determine the specificity of Cry proteins. Domain I is involved in membrane insertion and pore formation. Domain II is involved in specific receptor recognition and binding. Domain III maintains the structural integrity of the protein molecule (Li et al., 1991) and also contributes to specificity (De Maagd et al., 2001). The C-terminal domain is implicated in crystal formation, which does not directly contribute to the insecticidal activity (De Maagd et al., 2001). The C-terminal domain is cleaved upon entry into the insect midgut or by certain proteases *in vitro*. Only insects with specific receptors are affected and no toxicity is observed in species that lack these receptors (Crickmore et al., 1998; De Maagd et al., 2001). Please refer to **Section 2.5** for a more detailed explanation of the function of the Cry1A.105 and Cry2Ab2 proteins.

c) Proposed name the product will be marketed under

Maize (or corn) containing the transformation event MON 89034 will be marketed in North America as YieldGard VT Pro™. There are currently no plans to produce this product in Australia or New Zealand.

d) Products that are likely to include the food

Maize is used globally for food and feed. Food uses include sweet corn, popcorn, and processed field maize, which are all varieties / hybrids of *Zea mays* subsp. *mays*. Maize use globally is dominated by the use of field maize varieties / hybrids for animal feed (NCGA, 2000; Perry, 1988; Watson and Ramstad, 1987). Because of its high starch content, maize is used as a valuable energy source in animal feed for domestic livestock, such as cattle, pigs and poultry. Whole maize is usually ground and mixed with a high-protein feed compound and with vitamin and mineral supplements to balance the ration according to the nutritional requirements of the animals being fed (Leath and Hill, 1987; Watson and Ramstad, 1987).

Maize is also further processed into valuable food and industrial products. The primary product of wet milling is starch, a polymer made up of thousands of glucose units. Currently, the majority of the starch is converted to various sweeteners, while the remainder is consumed directly in foods and used for other industrial purposes. Much of the starch used for food and industrial uses is further chemically modified (*e.g.* bleaches, acids) and heat-treated to modify the starch properties to meet customer requirements (May, 1987).

Syrups derived from maize can be divided into regular syrups (*e.g.* glucose, dextrose), high fructose maize syrups and maltodextrins. A variety of enzymatic and acid-catalysed processes are used for the manufacture of refined sweeteners. Maize syrups are used in a wide array of foods and drugs to provide sweetness, viscosity or other enhancements. Ethanol is produced from starch by fermentation, which may be used in beverages or as a motor fuel supplement.

Maize oil is produced commercially from maize germ isolated by wet and dry milling and is actually a by-product of the maize milling industry. Oil is recovered from maize germ by expelling, solvent extraction, or a combination of the two processes. The resultant crude oil must be further refined, bleached and deodorised to produce good quality edible oil.

Typical products of the dry-milled fractions of degermed maize include flaking grits used for breakfast cereals, coarse grits used for cereals, regular grits used for cereals and snacks, coarse meal and dusted meal used for breads and muffins, flour used for pancakes, snacks, processed meat binder and oil.

The major by-products of the dry-milling industry are maize germ and bran. Maize bran has increased in popularity as a dietary source due to its desirable properties including low calorie content, high dietary fibre and high moisture retention. In

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addition, whole grain maize and maize grits are used to produce many distilled alcoholic beverages.

2.2 History of Use

a) Donor organism

The Cry1A.105 is a chimeric protein consisting of domains I and II from Cry1Ab or Cry1Ac[2], a substantial portion of domain III from Cry1F, and the C-terminal domain from Cry1Ac. Cry1Ac and Cry1Ab are derived from *Bacillus thuringiensis* subsp. *kurstaki* and Cry1F is derived from *Bacillus thuringiensis* subsp. *aizawai*. The Cry2Ab2 protein, also present in MON 89034 is derived from *Bacillus thuringiensis* subsp. *kurstaki*.

Bacillus thuringiensis (Bt) is a spore-forming, gram-positive bacterium that is found naturally in soil. Bt produces parasporal inclusions (i.e., crystals) during the stationary and sporulation phases of growth that contain proteins toxic to selected insect species. The appearance of parasporal inclusions distinguishes Bt from the other common soil bacterium, *Bacillus cereus*.

Bt has been used commercially over the last 40 years to control insect pests (McClintock et al., 1995; EPA, 1998). Corporate, institutional and government collections of Bt contain thousands of strain isolates from around the world. Several of these strain isolates have been extensively studied and commercialised as active ingredients for biopesticidal products (Baum et al., 1999). These products display selective insecticidal activity against a number of pests, including: *B. thuringiensis* subsp. *israelensis* strains that are active against dipteran insects (*e.g.*, mosquitoes and black flies); *B. thuringiensis* subsp. *tenebrionis* and *kumamotoensis* strains that are active against coleopteran insects (*e.g.*, corn rootworm, Colorado potato beetle, elm leaf beetle and yellow mealworm); and *B. thuringiensis* subsp. *kurstaki, thuringiensis, sotto* and *aizawai* strains that are active against lepidopteran insects (*e.g.*, European corn borer, tomato hornworm, gypsy moth, cabbage looper, tobacco budworm and corn earworm). Biopesticidal products based on recombinant Bt strains have also been commercialised for use in agriculture since the 1960's. Thus, the safety of Bt has been demonstrated by decades of use.

b) Host organism

i) Taxonomic classification

Maize (*Zea Mays* L.) is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae. The genera included in the tribe Maydae include *Zea* and *Tripsacum* in the Western Hemisphere, and *Coix, Polytoca, Chionachne, Schlerachne,* and *Trilobachne* in Asia. Although some researchers have implicated the Asian genera in the origin of maize, the evidence for them is not as extensive and convincing as for the genera located in the Western Hemisphere. **Table 1** summarises the taxonomic classification of maize and its close relatives. The genus *Zea* includes two sections: *Luxuriantes* and *Zea*. Corn (*Zea mays* L.) is a separate species within the subgenus *Zea*, along with three subspecies.

[2] Cry1Ab and Cry1Ac share 100% amino acid sequence identity at domains I and II.

Table 1. Taxonomic classification of maize and its close relatives

Family - Gramineae
- Subfamily - Panicoideae
  - Tribe - Maydae
    - Western Hemisphere:
      - I. Genus - *Zea*
        - A. Subgenus - *Luxuriantes*
          1. *Zea luxurians* (2n = 20)
          2. *Zea perennis* (2n = 40)
          3. *Zea diploperennis* (2n = 20)
        - B. Subgenus - *Zea*
          1. *Zea mays* (2n = 20)
             Subspecies
             1. *Z. mays parviglumis* (2n = 20)
             2. *Z. mays huehuetenangensis* (2n = 20)
             3. *Z. mays mexicana* (Schrad.) (2n = 20)

      - II. Genus – *Tripsacum*
        - A. Section – *Tripsacum*
          Species
          1. *T. andersomii* (2n = 64)
          2. *T. australe* (2n = 36)
             Varieties
             a) *T. australe* var. *australe*
             b) *T. australe* var. *hirstum*
          3. *T. bravum* (2n = 36, 72)
          4. *T. cundinamarce* (2n = 36) *guatemalense*
          5. *T. dactyloides* (2n = 72)
             Varieties
             a) *T. dactyloides* var. *hispidum*
             b) *T. dactyloides* var. *dactyloides*
             c) *T. dactyloides* var. *meridonale*
             d) *T. dactyloides* var. *mexicanum*
          6. *T. floridanum* (2n = 36)
          7. *T. intermedium* (2n = 72
          8. *T. manisuroides* (2n = 72)
          9. *T. latifolium* (2n = 36)
          10. *T. percuvianum* (2n = 72, 90, 108)
          11. *T. zopilotense* (2n = 36, 72)
        - B. Section – *Fasciculata*
          Species
          1. *T. jalapense* (2n = 72)
          2. *T. lanceolatum* (2n = 72)
          3. *T. fasciculatum* (2n = 36)
          4. *T. maizar* (2n = 36, 72)
          5. *T. pilosum* (2n = 72)
             Varieties
             a). *T. pilosum* var.
             b). *T. pilosum* var. *pilosum*

    - Asia:
      - I. Genera—
        Chionachne (2n = 20)
        Coix (2n = 10, 20)
        Polytoca (2n = 20)
        Schlerachne (2n = 20)
        Trilobachne (2n = 20)
  - Tribe—Andropogoneae
    - I. Genus - *Manisuris*

ii) Propagation of maize

Maize is an annual plant and the duration of its life cycle depends on the cultivars and on the environments in which the cultivars are grown (Hanway, 1966). Maize cannot survive temperatures below 0°C for more than six to eight hours after the growing point is above ground (five- to seven-leaf stage). Damage from freezing temperatures, however, depends on the extent of temperatures below 0°C, soil condition, amount of residue, the duration of freezing temperatures, wind movement, relative humidity, and stage of plant development. Light frosts in the late spring of temperate areas can cause leaf burning, but the extent of the injury usually is not great enough to cause permanent damage, although the maize crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. The completion of the life cycle of maize, therefore, is dictated by the duration of the average number of frost-free days.

The number of frost-free days dictates that maize with differences in the length of their life cycles can be grown in north-to-south directions of temperate areas. As an example, in the United States, maize with relative maturities of 80 days or less are grown in the extreme northern areas, and maize with relative maturities of more than 125 days are grown in the southern areas. Relative maturities, however, are not parallel lines east-to-west because they are dependent on prevailing weather patterns, topography, large bodies of water, and soil types (Troyer, 1994).

Maize has had a long history of safe use as a food since its introduction into the human diet. Maize originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus discovered the Western Hemisphere, maize was being grown by the indigenous civilisations from Chile to southern Canada. Columbus noted the presence of maize on the north coast of Cuba in November 1492, and introduced maize to Europe upon his return to Spain (Goodman, 1988). Within two generations after the introduction of maize to Europe, maize became distributed throughout those regions of the world where it could be cultivated.

Maize is widely consumed in Latin America, Asia, Africa, and the Balkans. In Latin America, maize is processed into tortillas, arepas, couscous, polenta, porridges, and various meals and gruels that are the basis for traditional foods (Rooney and Serna-Salvidar, 2003). In Africa and Asia, maize is generally dry milled into grits, meals and flours for the production of flat breads, steamed foods, snacks and alcoholic and non-alcoholic beverages.

iii) Maize processing

Maize is used globally for food and feed. Food uses include sweet corn, popcorn, and processed field maize, which are all varieties / hybrids of *Z. mays* subsp. *mays*. Maize use globally is dominated by the use of field maize varieties / hybrids for animal feed (NCGA, 2000; Perry, 1988; Watson and Ramstad, 1987). Because of its high starch content, maize is used as a valuable energy source in animal feed for domestic livestock, such as cattle, pigs and poultry. Whole maize is usually ground and mixed with high-protein feed compound and with vitamin and mineral supplements to balance the ration according to the nutritional requirements of the animals being fed

(Leath and Hill, 1987; Watson and Ramstad, 1987). Maize is also used for processing and the production of derivatives, which have a wide range of food, feed and industrial applications. Some of the processed fractions are used for animal feed, such as maize gluten, a resource that is rich in maize protein. Maize is also used for the production of feed additives.

Further, maize is processed into valuable food and industrial products, such as ethyl alcohol by fermentation, maize meal by dry milling, and highly refined starch by the wet milling process. The wet and dry mill processing of maize into refined derivatives (*e.g.* oil, syrups, starch, ethanol) involves varying degrees of mechanical, enzymatic, solvent, heat, acid, or pressure treatment or combinations of these steps.

The raw material used for wet milling is shelled field maize. However, some specialty maize types such as waxy and high-amylose maize that are contract-grown and identity-preserved are also used. Before the maize enters the production facility, it is inspected for the presence of fungal, insect, and rodent infestation (Freeman, 1973). Accepted maize is thoroughly cleaned by screening to remove pieces of cob, chaff, sand and other undesirable foreign matter (May, 1987; Watson, 1988). Dust and light chaff are removed by aspiration. The web milling process begins with the maize being steeped to soften the kernel for optimum milling conditions (Watson, 1988). The steep water is then drawn off and the softened kernels pass through an attrition (cracking) mill to liberate the germs. The germ fraction, containing approximately fifty percent oil on a dry weight basis, is separated from the denser components by flotation, and then washed and dried in preparation for oil recovery.

The remaining portion is finely milled maize endosperm containing starch and gluten (protein), and fibrous hulls (pericarps). The starch and gluten particles are separated from the fibre by passing the slurry over a series of screens. The starch and gluten are then separated, and the starch is washed and dried to be used as starch or converted into other products. High-protein gluten fractions are sold for animal feed formulations.

Maize is dry milled by one of two general processes, non-degerming and degerming. The non-degerming system stone-grinds the maize, preferably a white dent, to produce hominy grits and whole meals rich in bran and germ. However, the majority of maize is degermed during the dry milling (Serna-Salvidar et al., 1994). The degerming process targets as complete a separation of the maize parts as possible to (1) retain the maximum amount of horny endosperm portion as discrete pieces, (2) remove the maximum amount of germ and pericarp to give a low-fat, low-fibre product, and (3) recover the maximum number of intact germs (Alexander, 1987; Watson, 1988). The maize type most frequently used by millers has specific high density hard endosperm qualities (Paulsen et al., 2003). The degerming system produces highly refined grits, meals, and flours. As with wet milling, the maize is inspected for freedom from aflatoxin, insects, mould as well as some physical attributes of the kernels (Watson, 1987).

2.3 Characterisation of the introduced trait

a) Transformation method

MON 89034 was developed through *Agrobacterium*-mediated transformation of maize to produce the Bt insecticidal proteins Cry1A.105 and Cry2Ab2 using the binary plasmid vector, PV-ZMIR245 (**Figure 1**). *Agrobacterium*-mediated transformation is a well-documented process for the transfer and integration of exogenous DNA into a plant's nuclear genome. PV-ZMIR245 contains two separate T-DNAs (hereafter referred to as a 2 T-DNA system). The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette that encodes the neomycin phosphotransferase enzyme which confers tolerance to certain antibiotics such as neomycin and paromomycin.

The use of the 2 T-DNA system is the basis for an effective approach to generate marker-free plants. It allows for insertion of the T-DNA with the traits of interest (e.g., T-DNA I) and the T-DNA encoding the selectable marker (e.g., *nptII*, T-DNA II) into two independent loci within the genome of the plant. Following selection of the transformants, the inserted T-DNA encoding the selectable marker (e.g., T-DNA II) can be segregated from progeny through subsequent breeding and genetic selection, while the inserted T-DNA containing the trait(s) of interest is maintained (e.g., T-DNA I). This process is well documented (Depicker et al., 1985; De Framond et al., 1986; Komari et al., 1996; Yoder and Goldsbrough, 1994). Most recently, this approach was successfully used in transformation of barley (Matthews et al., 2001), rice (Breitler et al., 2004), soybean (Xing et al., 2000), and maize (Miller et al., 2002).

The maize germplasm that was utilised as the initial recipient of the transgenes in MON 89034 is the proprietary inbred line, LH172 (Eggerling, 1994). This inbred line was used because it responds well to transformation with *Agrobacterium* and tissue regeneration.

Freshly isolated immature maize embryos were used in the transformation (Ishida et al., 1996; Rout and Armstrong, 1997) that resulted in production of MON 89034. *Agrobacterium tumefaciens* strain ABI, containing plasmid PV-ZMIR245 was induced to be virulent by the use of acetosyringone. Strain ABI also contains a helper plasmid that does not contain any T-DNA but allows for the transfer of T-DNA I and T-DNA II to the plant cells. Following inoculation with *Agrobacterium*, the immature maize embryos were transferred to a co-culture medium for one to three days to ensure transformation of individual cells. This process of *Agrobacterium*-mediated transformation of maize involves the attachment of the bacterium to the maize cells, which leads to transfer of the region of DNA between the Left and Right Borders of the binary plasmid (i.e., the T-DNA) into the maize genomic DNA. Each T-DNA was integrated into the plant genome at separate loci.

Following the incubation period on the co-culture medium, the immature embryos were transferred to selection medium containing carbenicillin to eliminate *Agrobacterium*, and paromomycin to eliminate cells that were not transformed, so that

only cells containing T-DNA II and/or T-DNA I + T-DNA II survived. The resulting transformed cells were then subcultured several times on a selection medium and regenerated into the R_0 plants according to the protocol described by Armstrong and Phillips (1988).

During subsequent breeding at the F_1 generation the unlinked insertions of T-DNA I or T-DNA II were segregated. The plants containing only the insert that contains the *cry1A.105* and *cry2Ab2* gene cassettes were selected using molecular analysis, while the plants containing the *nptII* cassette (T-DNA II) were eliminated from subsequent breeding. The absence of the *nptII* gene and the NPTII protein was further confirmed by both Southern blot and ELISA analyses. **Figure 2** displays the process map of the major steps involved in the transformation, selection, and development of MON 89034.

b) Gene Construct and Vectors

MON 89034 was developed through *Agrobacterium*-mediated transformation of maize using the binary plasmid vector, PV-ZMIR245 (**Figure 1**). PV-ZMIR245 contains two separate T-DNAs. The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette that encodes the neomycin phosphotransferase enzyme that confers tolerance to certain antibiotics such as neomycin and paromomycin. The following description on the different elements present on plasmid PV-ZMIR245 is supported by **Table 2**.

i) T-DNA I

- **The *cry1A.105* gene**

The *cry1A.105* coding sequence encodes the 133 kDa Cry1A.105 insecticidal protein that provides protection against feeding damage by lepidopteran insect pests. The Cry1A.105 is a modified *Bt* Cry1A protein with amino acid sequence identity to Cry1Ab, Cry1Ac and Cry1F proteins of 90.0%, 93.6% and 76.7%, respectively.

- **The *cry1A.105* regulatory sequences**

The expression cassette for the coding sequence of the Cry1A.105 protein consists of the promoter (P-e35S) and leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing a duplicated enhancer region (Kay et al., 1987). It contains the 5' untranslated leader of the wheat chlorophyll a/b/ binding protein (L-*Cab*) (Lammpa et al., 1985), the intron from the rice acting gene (*I-Ract1*) (McElroy et al., 1991), the *cry1A.105* coding sequence that was optimised for the expression in monocots, and the 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3 (T-*Hsp17*), which terminates transcription and provides the signal for mRNA polyadenylation (McElwain and Spiker, 1989).

- **The *cry2Ab2* gene**

The Cry2Ab2 protein present in MON 89034 is a member of the Cry2Ab class of proteins that share >95% amino sequence homology (Crickmore et al., 1998). It is a variant of the wild-type Cry2Ab2 protein isolated from Bt (subsp. *kurstaki*).

- **The *cry2Ab2* regulatory sequences**

The *cry2Ab2* gene expression cassette that produces the Cry2Ab2 protein consists of the 35S promoter from figwort mosaic virus (P-*FMV*; Rogers, 2000), the first intron from the maize heat shock protein 70 gene (I-*Hsp* 70; Brown and Santino, 1995). It contains a *cry2Ab2* coding sequence with a modified codon usage (CS-*cry2Ab2*; Widner and Whitely, 1989; Donovan, 1991) fused to a chloroplast transit peptide region of maize ribulose 1,5-biphosphate carboxylase small subunit including the first intron (TS-*SSU-CTP*; Matsuoka et al., 1987). The 3' nontranslated region of the nopaline synthase (T-*nos*) coding region from *Agrobacterium tumefaciens* T-DNA terminates transcription and directs polyadenylation (Bevan et al., 1983).

- **T-DNA borders**

Plasmid vector PV-ZMIR245 contains sequences that are necessary for transfer of T-DNA into the plant cell. These sequences are termed the Right and Left Border regions. The Right and Left Border regions each contains a border sequence that is a 24-26 bp sequence that defines the extent of the DNA that should be transferred into the plant genome. They flank both T-DNA I and T-DNA II, allowing for independent transfer and integration of each T-DNA into the plant genome during transformation. The Right Borders present in PV-ZMIR245 are made of a 24 bp nucleotide sequence that was originally derived from plasmid pTiT37 which was isolated from *A. tumefaciens* (Depicker et al., 1982). The Left Borders present in PV-ZMIR245 are made of a 25 bp nucleotide sequence from the *A. tumefaciens* plasmid pTi5955, a derivative of plasmid pTiA6 (Barker et al., 1983).

ii) T-DNA II

- ***nptII* gene**

The *nptII* gene encodes the neomycin phosphotransferase II enzyme (NPTII) that inactivates certain aminoglycoside antibiotics such as kanamycin, neomycin and paromomycin. The use of selectable marker genes, such as *nptII*, is essential to select transformed cells under selective growth conditions. In the presence of paromomycin; cells transformed with *nptII* survive, while those that do not contain *nptII* are removed due to the action of the paromomycin. The T-DNA II, and therefore the *nptII* gene, is segregated out at the F_1 generation (see **Section 2.3.a.**).

- ***nptII* regulatory sequences**

The *nptII* gene cassette that produces the NPTII protein consists of the promoter (P-*e35S*) from the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985). The sequence coding for the NPTII protein (Beck et al., 1982) is followed by the 3' nontranslated region of the nopaline synthase (T-*nos*) coding region from

Agrobacterium tumefaciens T-DNA that ends transcription and directs polyadenylation (Bevan et al., 1983).

- **T-DNA borders**

The right and left T-DNA borders are described under **Section 2.3.b.i.**

iii) Genetic elements outside the T-DNA borders

The backbone region outside of the inserted DNA, contains two origins of replication necessary for replication and maintenance of the plasmid in bacteria. In addition, it contains a bacterial selectable marker gene, *aad*, which encodes an aminoglycoside-modifying enzyme that confers resistance to the action of the antibiotics spectinomycin and streptomycin. Detailed descriptions of all elements in the plasmid backbone region are presented in **Table 2**. The absence of the backbone sequence in MON 89034 has been confirmed by Southern blot analyses, which are presented in the following section.

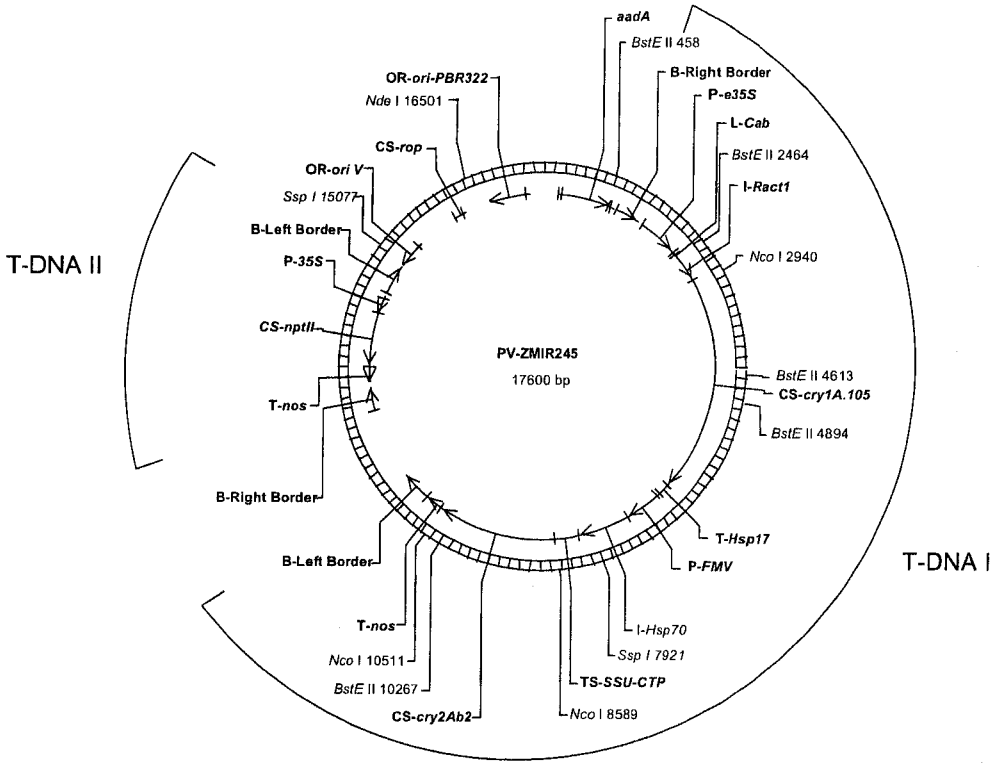


Figure 1. Plasmid Map of Vector PV-ZMIR245

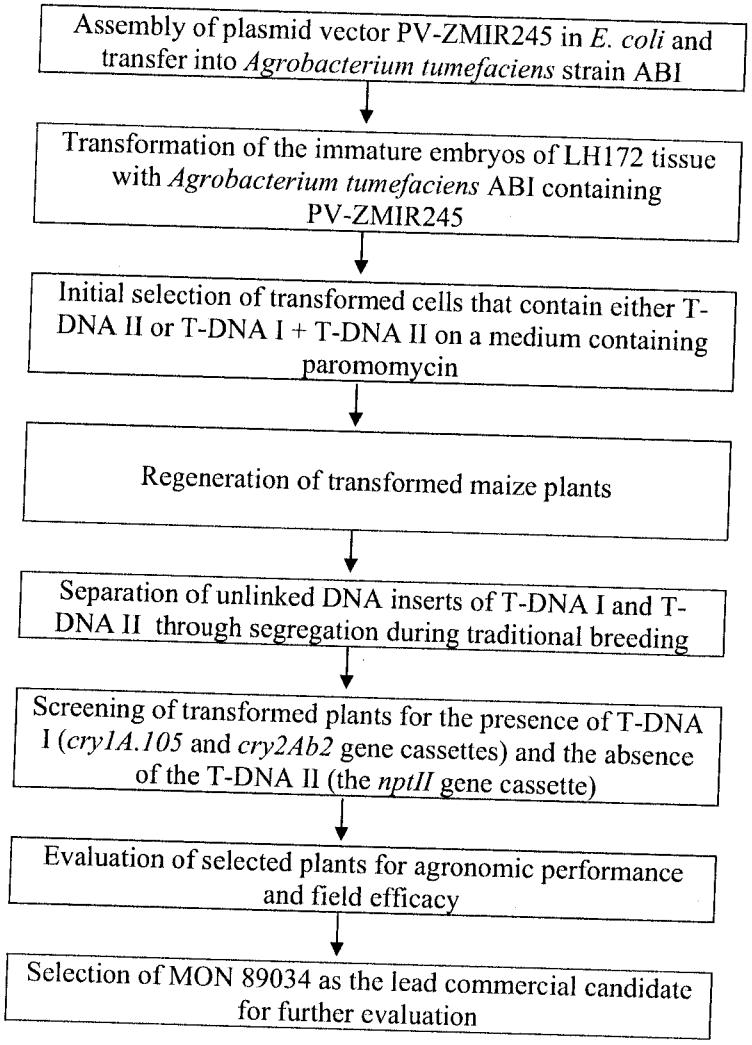


Figure 2. Process Map for Transformation, Selection, Regeneration and Evaluation of MON 89034

Table 2. Summary of genetic elements in vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Vector Backbone		
Intervening Sequence	1-257	Sequence used in DNA cloning.
aadA	258-1146	Bacterial promoter, coding sequence, and terminator sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al. 1985) (GenBank accession X03043). *aadA* confers resistance to streptomycin and spectinomycin.
Intervening Sequence	1147-1261	Sequence used in DNA cloning.
T-DNA I		
B[3]-Right Border	1262-1618	DNA region from *Agrobacterium tumefaciens* containing the 24 bp right border sequence used for transfer of the T-DNA (Depicker et al., 1982).
Intervening Sequence	1619-1728	Sequence used in DNA cloning.
P[4]-*e35S*	1729-2349	Promoter and 9bp leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987).
Intervening Sequence	2350-2375	Sequence used in DNA cloning.
L[5]-*Cab*	2376-2436	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al., 1985).
Intervening Sequence	2437-2452	Sequence used in DNA cloning.
I[6]-*Ract1*	2453-2932	Intron from the rice actin gene (McElroy et al., 1991).
Intervening Sequence	2933-2941	Sequence used in DNA cloning.
CS[7]-*cry1A.105*[8]	2942-6475	Coding sequence for the *Bacillus thuringiensis* Cry1A.105 protein (Monsanto unpublished data).

[3] B – border region
[4] P - promoter
[5] L - leader
[6] I - intron
[7] CS – coding sequence
[8] Coding sequence of the *cry1A.105* includes stop codon

Table 2 (cont.) Summary of genetic elements in vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	6476-6506	Sequence used in DNA cloning.
T[9]-*Hsp17*	6507-6716	3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation (McElwain and Spiker, 1989).
Intervening Sequence	6717-6783	Sequence used in DNA cloning.
P-*FMV*	6784-7347	Figwort Mosaic Virus 35S promoter (Rogers, 2000).
Intervening Sequence	7348-7369	Sequence used in DNA cloning.
I-*Hsp70*	7370-8173	First intron from the maize heat shock protein 70 gene (Brown and Santino, 1995).
Intervening Sequence	8174-8189	Sequences used in DNA cloning.
TS*[10]*-SSU-CTP	8190-8590	DNA region containing the targeting sequence for the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit and the first intron (Matsuoka et al., 1987).
CS-*cry2Ab2*[11]	8591-10498	Coding sequence for a Cry2Ab2 protein from *Bacillus thuringiensis* (Widner and Whitely, 1989; Donovan, 1991). This coding sequence uses a modified codon usage.
Intervening Sequence	10499-10524	Sequence used in DNA cloning.
T-*nos*	10525-10777	3' transcript termination sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983).
Intervening Sequence	10778-10844	Sequence used in DNA cloning.
B-Left Border	10845-11286	DNA region from *Agrobacterium tumefaciens* containing the 25 bp left border sequence used for transfer of the T-DNA (Barker et al., 1983).

[9] T – transcript termination sequence
[10] TS – targeting sequence
[11] The *cry2Ab2* coding sequence includes two stop codons

Table 2 (cont.) Summary of genetic elements in vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
		Vector Backbone
Intervening Sequence	11287-12489	Sequence used in DNA cloning.
		T-DNA II
B-Right Border	12490-12846	DNA region from *Agrobacterium tumefaciens* containing the 24 bp right border sequence used for transfer of the T-DNA (Depicker et al., 1982).
Intervening Sequence	12847-12971	Sequence used in DNA cloning.
T-*nos*	12972-13224	3' termination sequence of the nopaline synthase (*nos*) coding sequence from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983).
Intervening Sequence	13225-13255	Sequence used in DNA cloning.
CS-*nptII*	13256-14050	Coding sequence for neomycin phosphotransferase II protein that confers resistance to neomycin and kanamycin (Beck et al., 1982).
Intervening Sequence	14051-14083	Sequence used in DNA cloning.
P-*35S*	14084-14407	Promoter and 31bp leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985).
Intervening Sequence	14408-14457	Sequence used in DNA cloning.
B-Left Border	14458-14899	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983).
		Vector Backbone
Intervening Sequence	14900-14985	Sequence used in DNA cloning.
OR-*ori V*	14986-15382	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker et al., 1981).

Table 2 (cont.) Summary of genetic elements in plasmid vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	15383-16119	Sequence used in DNA cloning.
CS-*rop*	16120-16311	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).
Intervening Sequence	16312-16738	Sequence used in DNA cloning.
OR-*ori-PBR322*	16739-17327	Origin of replication from pBR322 allowing maintenance of plasmid in *E. coli* (Sutcliffe, 1978).
Intervening Sequence	17328-17600	Sequence used in DNA cloning.

c) Molecular Characterisation

Molecular analysis was performed to characterise the inserted DNA in MON 89034. This analysis was performed to confirm that only the DNA associated with the desired trait is present in the final product.

Specifically, Southern blot analyses were performed to assess:

a) Number of inserts in the genome,
b) Number of copies of insert,
c) Intactness of the genetic elements within the insert,
d) Absence of backbone sequences,
e) Absence of the T-DNA II encoding the selectable marker,
f) Stability of the inserted DNA across multiple generations, and
g) Organisation of the insert.

The information and results derived from the molecular analyses were used to construct a linear map of the insert in MON 89034. This linear map depicts restriction sites identified in the insert and the flanking maize genome, and provides information on the expected banding patterns and sizes of the DNA fragments after restriction enzyme digestions. The linear map is shown in **Figure 3**.

i) Insert and Copy Number Determination

Southern blot analyses were performed to assess insert and copy number of the DNA inserted in MON 89034. **Figures 4** and **5** show the complete set of probes used in the Southern blot analyses. **Figure 6** shows the Southern blot result on insert and copy number determination. The insert number (the number of integration sites of T-DNA I in the maize genome) was evaluated by digesting the test and control DNA with *Nde* I, a restriction enzyme that does not cleave within T-DNA I. This enzyme generates a restriction fragment containing T-DNA I and adjacent, plant genomic DNA. Thus, the number of restriction fragments detected indicates the number of inserts present in MON 89034. The electrophoresis of the digested DNA was conducted to achieve both resolution of high molecular weight fragments (long run) and to allow visualisation of smaller molecular size bands (short run).

The number of copies of the T-DNA present was determined by digesting test and control genomic DNA samples with *Ssp* I, which cleaves once within the insert. If MON 89034 contains one copy of the insert, probing with overlapping T-DNA I [probes 18 – 23 in **Figure 5**] should result in two bands, each representing a portion of the insert along with adjacent, plant genomic DNA.

Figure 6 shows that genomic DNA isolated from conventional maize digested with *Nde* I (lanes 2 and 6) or *Ssp* I (lanes 4 and 8) produced several hybridisation signals. This was expected because several of the genetic elements comprising T-DNA I were originally were derived from maize (see **Table 2**). These hybridisation signals result from the probes hybridising to endogenous targets residing in the conventional maize genome and are not specific to the inserted DNA. These signals were produced in all lanes, including those containing the conventional maize control DNA material, and

therefore they are considered to be endogenous background. Plasmid PV-ZMIR245 DNA mixed with conventional maize control DNA and digested with *Ssp I* (lanes 9 and 10) produced the expected bands at ~10.4 and ~7.2 kb in addition to the endogenous background hybridisation produced by the conventional maize control DNA (lane 8).

MON 89034 DNA digested with *Nde* I (lanes 1 and 5) produced a single unique band of ~13 kb in addition to the endogenous background hybridisation observed in the conventional maize control DNA (lanes 2 and 6). This result confirms that MON 89034 contains one insert located on ~13 kb *Nde* I restriction fragment.

The MON 89034 DNA digested with *Ssp* I (lanes 3 and 7) produced two bands in addition to the endogenous background hybridisation observed in the conventional maize control DNA (lanes 4 and 8). The ~8.2 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (corresponding to T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (**Figure 3**). The ~7.4 kb band, which was expected to be >4.3 kb, represents the 3' border fragment containing the 3' end of the inserted DNA along with the adjacent genomic DNA flanking the 3' end of the insert.

The results presented in **Figure 6** indicate that MON 89034 contains one copy of T-DNA I that resides at a single locus of integration on ~13 kb *Nde* I restriction fragment.

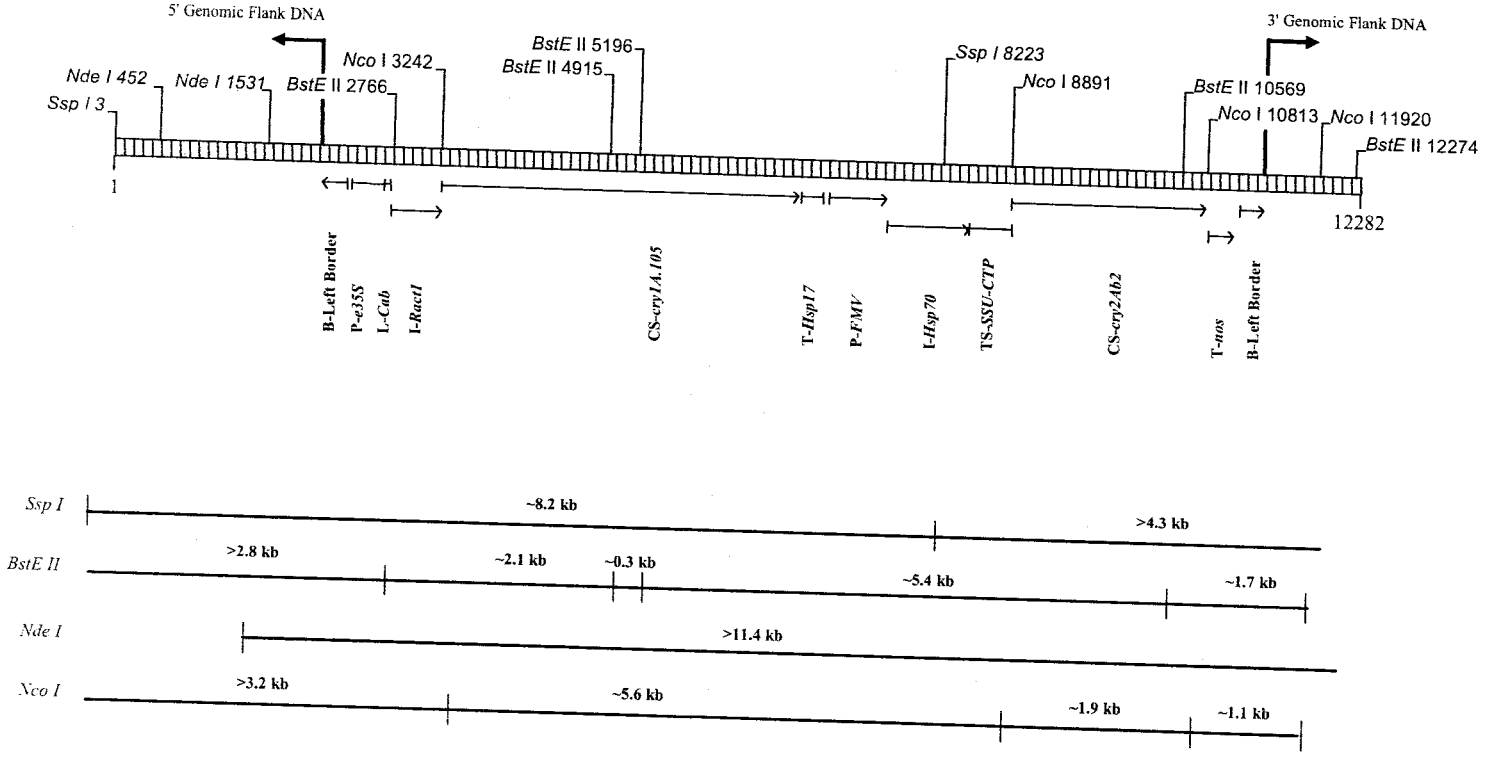
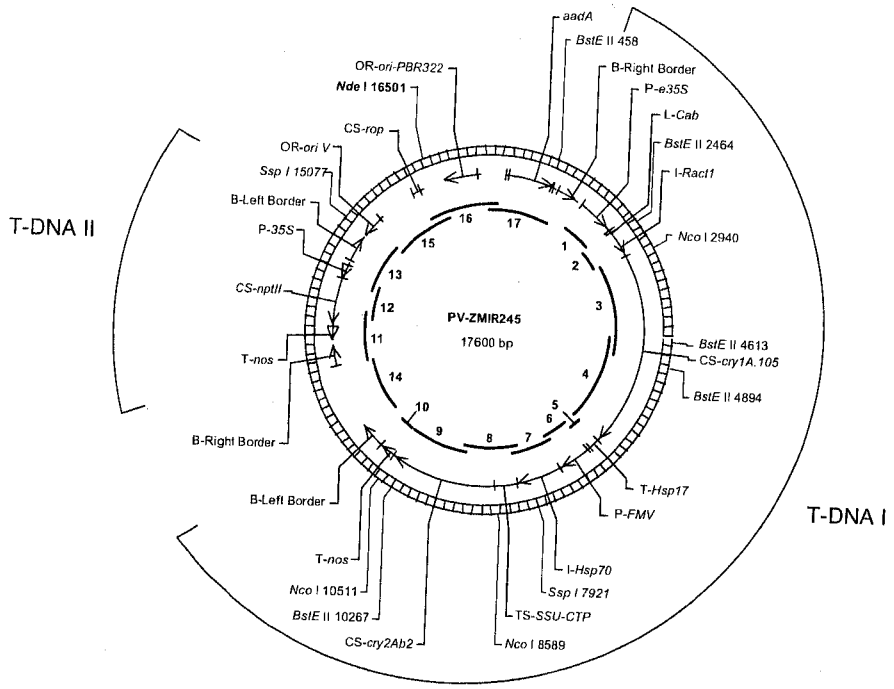


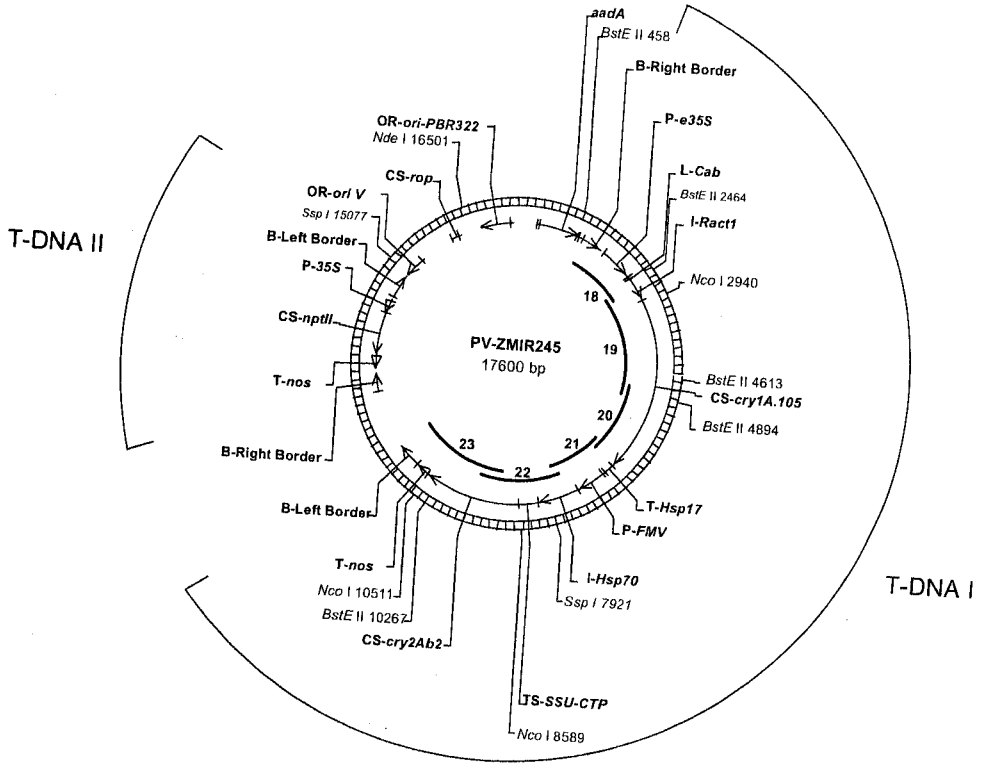
Figure 3. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 89034
The linear DNA derived from T-DNA I of vector PV-ZMIR245, which was incorporated into MON 89034 is shown. Arrows indicate the end of the insert and the beginning of maize genomic flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern blot analyses. A portion of Left Border sequence and a modified *e35S* promoter sequence is present at the 5' insert-to-flank junction in MON 89034.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	P-*e35S*/L-*Cab*	1714	2447	0.7
2	I-*Ract1*	2427	2941	0.5
3	CS-*cry1A.105* probe 1	2942	4923	2.0
4	CS-*cry1A.105* probe 2	4726	6505	1.8
5	T-*Hsp17*	6490	6797	0.3
6	P-*FMV*	6755	7366	0.6
7	I-*Hsp70*	7347	8179	0.8
8	TS-*SSU-CTP*/CS-*cry2Ab2* probe 1	8173	9516	1.3
9	TS-*SSU-CTP*/CS-*cry2Ab2* probe 2	9296	10509	1.2
10	T-*nos*	10525	10778	0.3
11	T-DNA II probe 1	12458	13391	0.9
12	T-DNA II probe 2/CS-*nptII* probe	13256	14050	0.8
13	T-DNA II probe 3	13973	14916	0.9
14	Backbone 1	11287	12489	1.2
15	Backbone 2	14900	16511	1.6
16	Backbone 3	16289	136	1.4
17	Backbone 4	48	1261	1.2

Figure 4. Genetic elements and restriction sites of vector PV-ZMIR245 used in Southern blot analyses (Probes 1-17)

A circular map of the plasmid vector PV-ZMIR245 used to develop maize MON 89034 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map. PV-ZMIR245 contains two T-DNA regions designated as T-DNA I and T-DNA II.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
18	T-DNA I probe 1	1210	2753	1.5
19	T-DNA I probe 2	2649	4676	2.0
20	T-DNA I probe 3	4518	6505	2.0
21	T-DNA I probe 4	6371	8179	1.8
22	T-DNA I probe 5	8004	9863	1.9
23	T-DNA I probe 6	9780	11354	1.6

Figure 5. Genetic elements and restriction sites of vector PV-ZMIR245 used in Southern blot analyses (Probes 18 - 23)

A circular map of the plasmid vector PV-ZMIR245 used to develop maize MON 89034 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The overlapping T-DNA I probes used in the Southern analyses are shown on the interior of the map.

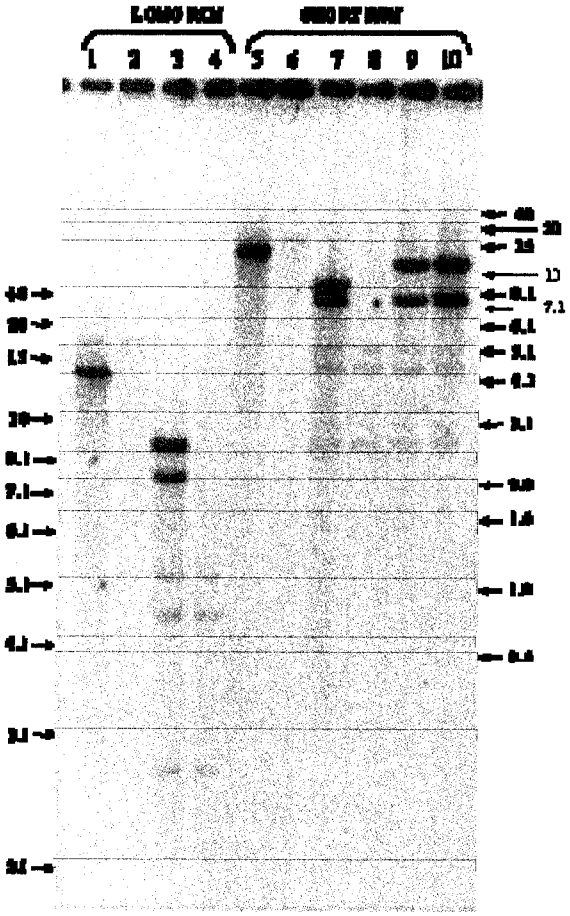


Figure 6. Southern blot analysis of MON 89034: insert and copy number
The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 - 23, **Figure 5**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Nde* I)
2: Conventional maize (*Nde* I)
3: MON 89034 (*Ssp* I)
4: Conventional maize (*Ssp* I)
5: MON 89034 (*Nde* I)
6: Conventional maize (*Nde* I)
7: MON 89034 (*Ssp I*)
8: Conventional maize (*Ssp I*)
9: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

ii) Intactness of the *cry1A.105* and *cry2Ab2* Expression Cassettes

The presence and intactness of all the elements of the inserted *cry1A.105* and *cry2Ab2* expression cassettes was assessed by digestion of MON 89034 genomic DNA with the restriction enzyme *Ssp* I, *Nco* I, or *Bst*E II. This is necessary to ensure that only expected proteins are produced in MON 89034.

Digestion with *Ssp* I releases two border fragments with the expected size of ~8.2 and >4.3 kb (**Figure 3**). The ~8.2 kb fragment contains genomic DNA flanking the 5' end of the insert, Left Border sequence, modified *e35S* promoter sequence, *Cab* leader, *Ract1* intron, *cry1A.105* coding sequence, *Hsp17* 3' end sequence, *FMV* promoter, and a portion of the *Hsp70* intron. The border fragment >4.3 kb contains the remaining portion of the *Hsp70* intron, *SSU-CTP* targeting sequence, *cry2Ab2* coding sequence, *nos* 3' end sequence, Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA (**Figure 3**).

Digestion of MON 89034 genomic DNA with *Nco* I releases two internal restriction fragments and two border fragments (**Figure 3**). The 5' border fragment is expected to be >3.2 kb and contains genomic DNA flanking the 5' end of the insert, the Left Border sequence, modified *e35S* promoter sequence, the *Cab* leader, and the *Ract1* intron. The ~5.6 kb internal fragment contains the *cry1A.105* coding sequence, *Hsp17* 3' end sequence, *FMV* promoter, *Hsp70* intron, and the *SSU-CTP* targeting sequence. The ~1.9 kb internal fragment contains the *cry2Ab2* coding sequence. The 3' border fragment is expected to be ~1.1 kb and contains the *nos* 3' end sequence, a second Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA.

Digestion of MON 89034 genomic DNA with *Bst*E II generates two border fragments and three internal fragments (**Figure 3**). The 5' border fragment is expected to be >2.8 kb and contains genomic DNA flanking the 5' end of the insert, Left Border sequence, modified *e35S* promoter sequence, and the *Cab* leader sequence. The 3' border fragment is expected to be ~1.7 kb and contains a portion of the *cry2Ab2* coding sequence, the *nos* 3' end, Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA.

Plasmid PV-ZMIR245 DNA was combined with conventional maize control DNA and digested with *Nco* I or *Bst*E II (*Bst*E II was used for the T-*nos* and T-DNA II Southern blots) and loaded on the gel to serve as a positive hybridisation control.

Individual Southern blots were examined with the following probes: *e35S* promoter including the *Cab* leader, the *Ract1* intron, the *cry1A.105* coding sequence, the *Hsp17* 3' end sequence, the *FMV* promoter, the *Hsp70* intron, the *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence, and the *nos* 3' end sequence (probes 1-10, **Figure 4**).

- ***e35S* promoter/*Cab* leader**

The results of this analysis are presented in **Figure 7**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) probed with the *e35S* promoter and *Cab* leader probe showed no hybridisation bands, as expected for the negative control. Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~10 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the single unique band of ~5.4 kb. This is consistent with the expected band being >3.2 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *e35S* promoter and *Cab* leader elements other than those associated with the *cry1A.105* cassette.

- ***Ract1* intron**

The results of this analysis are presented in **Figure 8**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) probed with the *Ract1* intron probe showed no hybridisation bands, as expected for the negative control.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~10 kb. The migration of the ~10 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the maize DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the single unique ~ 5.4 kb band. This is consistent with the expected band being >3.2 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *Ract1* intron elements other than those associated with the *cry1A.105* cassette.

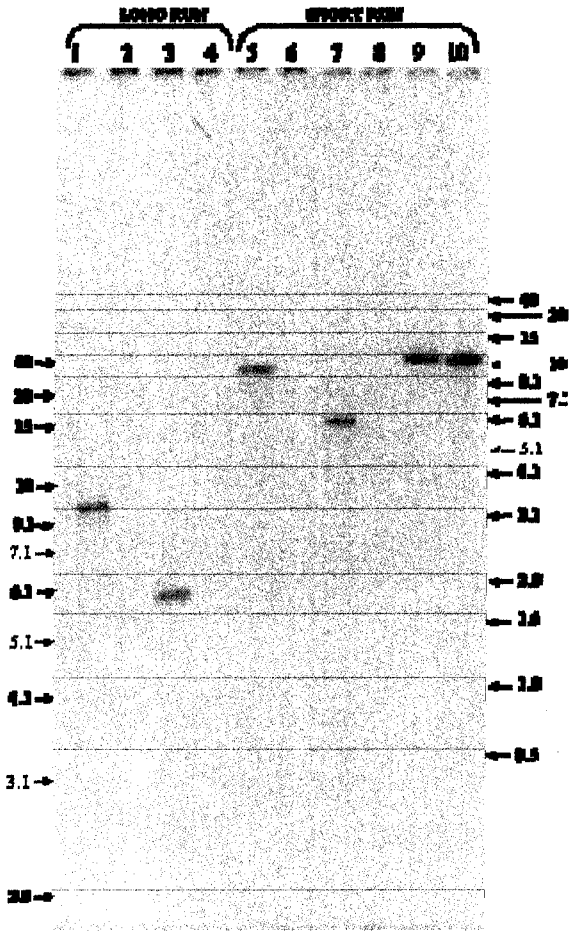


Figure 7. Southern blot analysis of MON 89034: *e35S* promoter/*Cab* leader
The blot was examined with a ^{32}P-labeled probe that spanned the *e35S* promoter and *Cab* leader region (probe 1, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

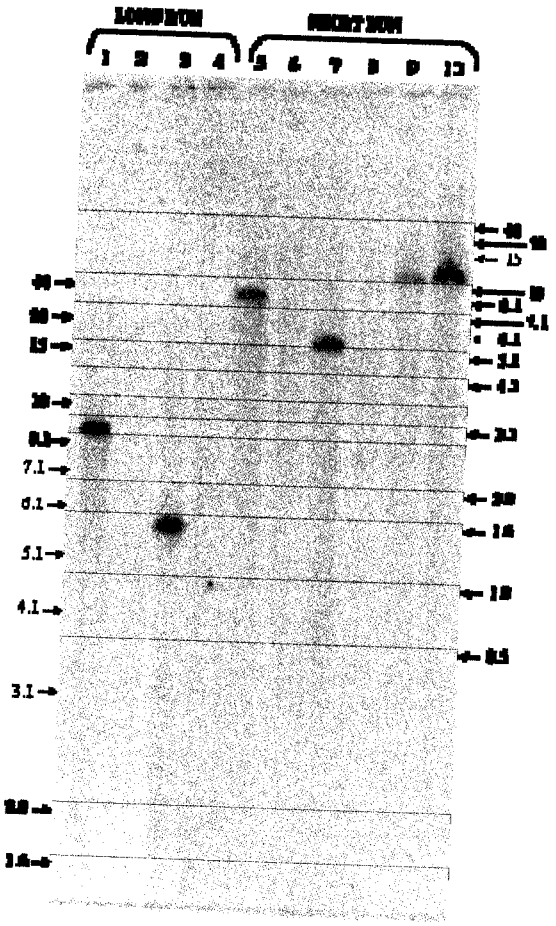


Figure 8. Southern blot analysis of MON 89034: *Ract1* intron
The blot was examined with a ^{32}P-labeled probe that spanned the *Ract1* intron (probe 2, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

- ***cry1A.105* coding sequence**

The results of this analysis are presented in **Figure 9**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) hybridised simultaneously with overlapping probes spanning the *cry1A.105* coding sequence showed no hybridisation bands as expected for the negative control. Conventional maize DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb. The migration of the ~ 5.6 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the maize DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected single unique band of ~5.6 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *cry1A.105* elements other than those associated with the *cry1A.105* cassette.

- ***Hsp17* 3' end sequence**

The results of this analysis are presented in **Figure 10**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *Hsp17* 3' end sequence probe showed no hybridisation bands, as expected for the negative control. Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected single unique band of ~5.6 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *Hsp17* 3' end elements other than those associated with the *cry1A.105* cassette.

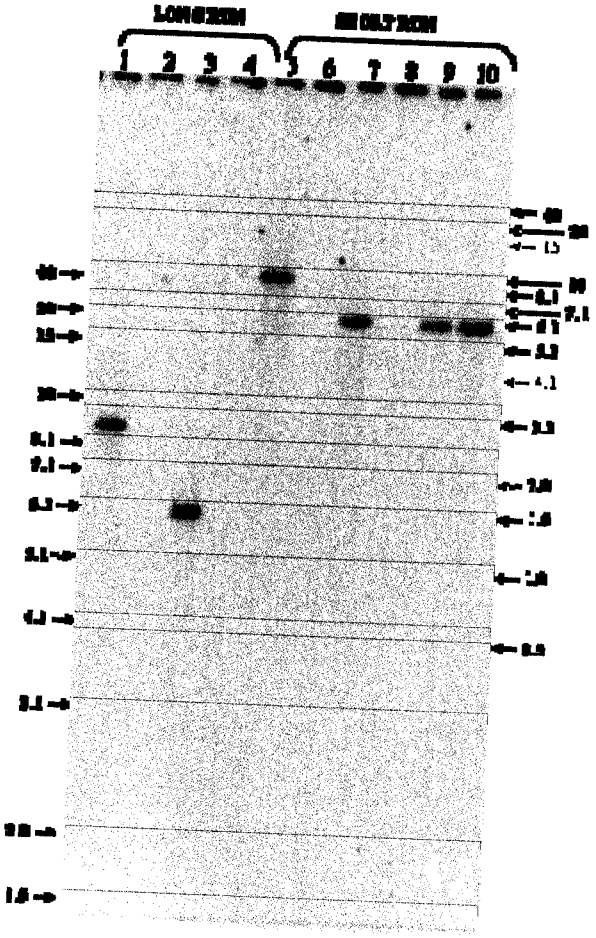


Figure 9. Southern blot analysis of MON 89034: *cry1A.105* coding sequence
The blot was examined with overlapping ^{32}P-labeled probes that spanned the *cry1A.105* coding sequence (probes 3 and 4, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

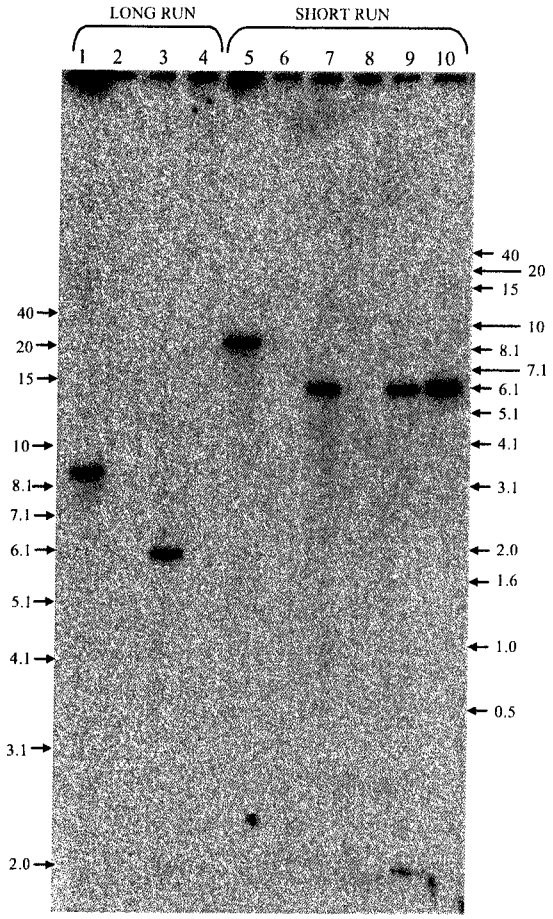


Figure 10. Southern blot analysis of MON 89034: *Hsp17*3' end sequence
The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp17* 3' end sequence (probe 5, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

- ***FMV* promoter**

The results of this analysis are presented in **Figure 11**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *FMV* promoter probe showed no hybridisation bands, as expected for the negative control.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb. The migration of the ~5.6 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the maize DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected single unique band of ~5.6 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *FMV* elements other than those associated with the *cry2Ab2* cassette.

- ***Hsp70* intron**

The results of this analysis are presented in **Figure 12**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *Hsp70* intron probe produced several hybridisation signals. This is not unexpected because the *Hsp70* intron was originally derived from maize. These hybridisation signals result from the probes hybridising to endogenous sequences residing in the maize genome and are not specific to the inserted DNA. These signals were produced in both test and control lanes, and therefore the bands are considered to be endogenous background.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb in addition to the endogenous bands. The migration of the ~5.6 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the maize DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced two expected bands of ~8.2 and ~7.4 kb in addition to the endogenous bands. The ~8.2 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA 1) along with the adjacent genomic DNA flanking the 5' end of the insert (see **Figure 3**). The ~7.4 kb band represents the 3' border fragment containing the 3' end of the inserted DNA along with the adjacent genomic DNA flanking the 3' end of the insert which was expected to be >4.3 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected band of ~5.6 kb in addition to the endogenous bands that resulted from non-specific hybridisation as shown on lanes 4 and 8. No unexpected bands were detected, indicating that MON 89034 contains no additional *Hsp70* intron elements other than those associated with the *cry2Ab2* cassette.

- ***SSU-CTP* targeting sequence/*cry2Ab2* coding sequence**

The results of this analysis are presented in **Figure 13**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) and examined with overlapping probes spanning the *SSU-CTP/cry2Ab2* coding sequence probe (probes 8 and 9, **Figure 4**) produced several hybridisation signals. This is expected because the *SSU-CTP* targeting sequence was originally derived from maize. These hybridisation signals result from the probes hybridising to endogenous targets residing in the maize genome and are not specific to the inserted DNA. These signals were produced with both test and control material, therefore they are considered to be endogenous background. Endogenous bands were not detected in the long runs of the *Nco* I digests because they ran off the gel, as expected.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size bands at ~1.9 and ~5.6 kb in addition to the endogenous bands.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced one expected band of ~7.4 kb in addition to the endogenous bands. The ~7.4 kb band is consistent with the expected band of >4.3 kb (see **Figure 3**). MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced two bands in addition to the endogenous bands that are consistent with the expected sizes of ~5.6 and ~1.9 kb. The position of the these bands was slightly higher than indicated by the molecular marker band sizes in the long run (lane 3) but was concurrent with the bands produced by PV-ZMIR245 in the short run (lanes 7, 9, and 10). The altered migrations may be due to the difference in salt concentrations between the maize DNA sample and the molecular marker (Sambrook and Russell, 2001). No unexpected bands were detected, indicating that MON 89034 contains no additional *SSU-CTP/cry2Ab2* elements other than those associated with the *cry2Ab2* cassette.

- **T-*nos* sequence**

The results of this analysis are presented in **Figure 14**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Bst*E II (lanes 4 and 8) examined with the *nos* 3' end sequence probe showed no detectable hybridisation bands, as expected for the negative control. Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Bst*E II (lanes 9 and 10) produced the expected size band at ~7.8 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced a single, unique band of ~7.4 kb that is consistent with the expected band >4.3 kb (see **Figure 3**). MON 89034 DNA digested with *Bst*E II (lanes 3 and 7) produced the expected single unique band of ~1.7 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *nos* 3' end elements other than those associated with the *cry2Ab2* cassette.

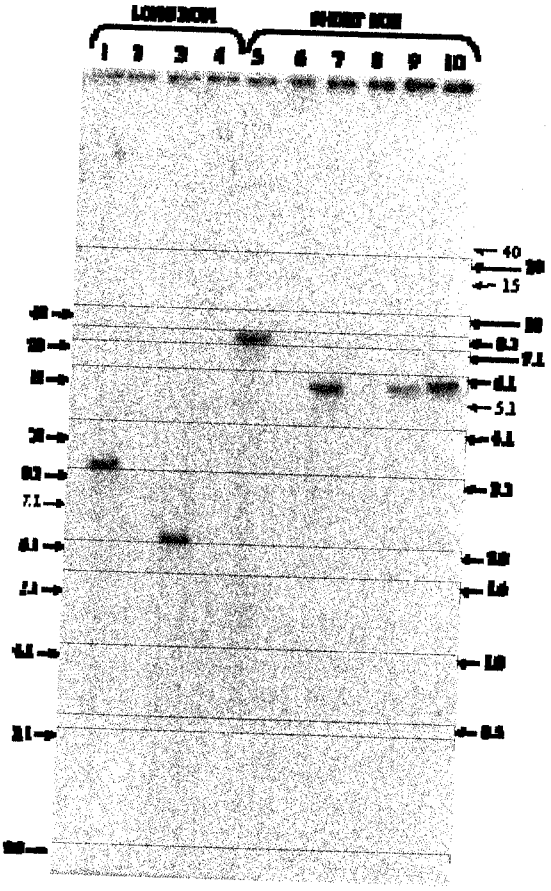


Figure 11. Southern blot analysis of MON 89034: *FMV* promoter
The blot was examined with a ^{32}P-labeled probe that spanned the *FMV* promoter (probe 6, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

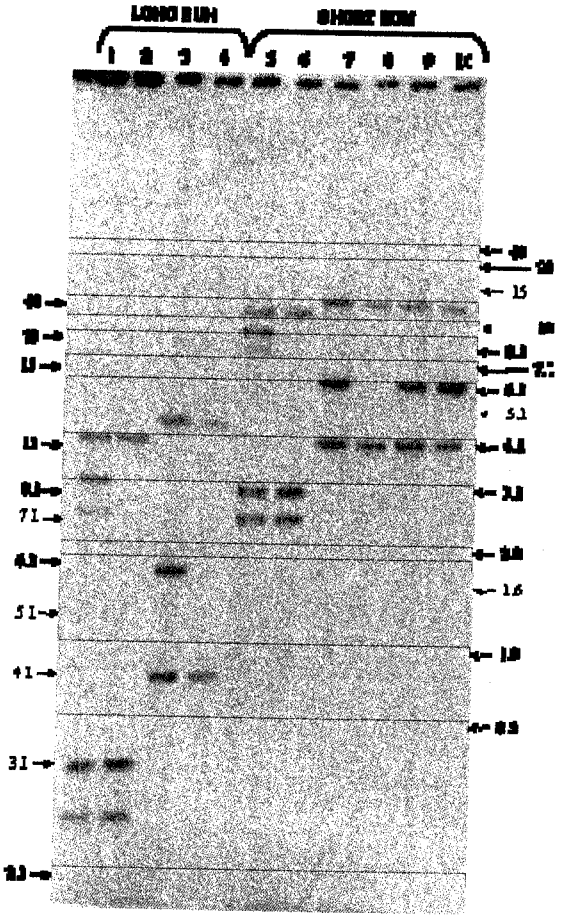


Figure 12. Southern blot analysis of MON 89034: *Hsp70* intron

The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp70* intron (probe 7, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

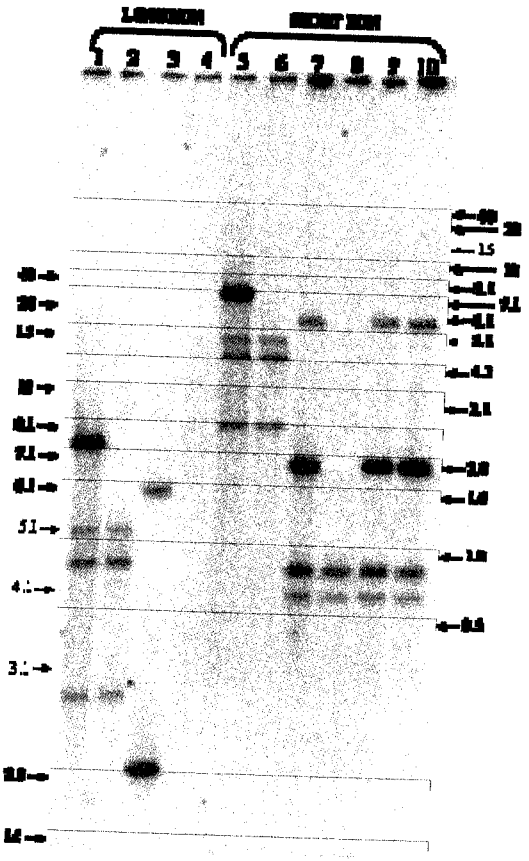


Figure 13. Southern blot analysis of MON 89034: *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence

The blot was examined with overlapping ^{32}P-labeled probes that spanned the *SSU-CTP* targeting sequence and *cry2Ab2* coding sequence (probes 8 and 9, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

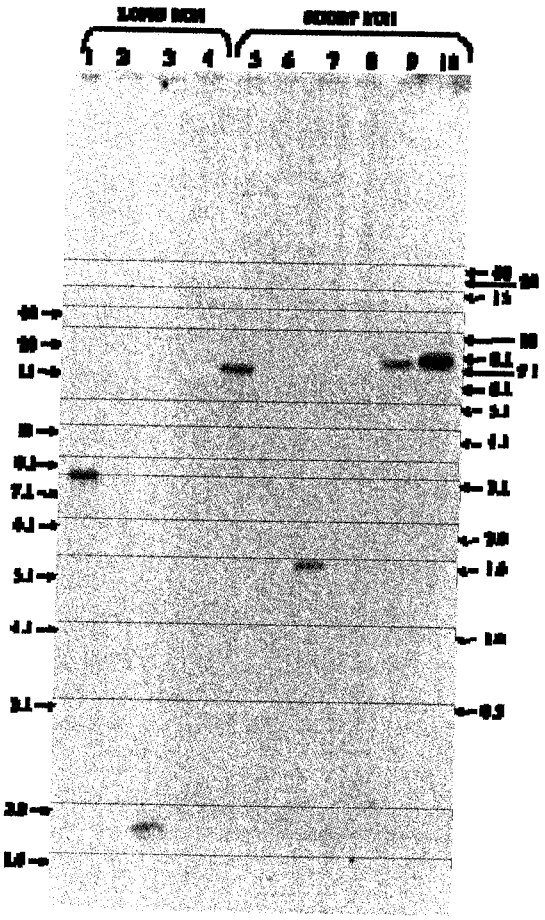


Figure 14. Southern blot Aanalysis of MON 89034: *nos* 3' end sequence
The blot was examined with a ^{32}P-labeled probe that spanned the *nos* 3' end sequence (probe 10, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Bst*E II)
4: Conventional maize (*Bst*E II)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Bst*E *II*)
8: Conventional maize (*Bst*E *II*)
9: Conventional maize spiked with PV-ZMIR245 (*Bst*E II) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Bst*E II) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

iii) Confirmation of the Absence of Plasmid PV-ZMIR245 Backbone

Southern blot analysis was used to confirm that the plasmid backbone sequences necessary for transformation are no longer present in MON 89034. This is important to ensure that only desired proteins are produced in MON 89034.

The results of this analysis are presented in **Figure 15**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with overlapping probes spanning the vector backbone of PV-ZMIR245 showed no hybridisation bands as expected for the negative control.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of ~10 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) of *Nco* I (lanes 3 and 7) produced no detectable hybridisation bands, indicating that MON 89034 contains no PV-ZMIR245 backbone elements.

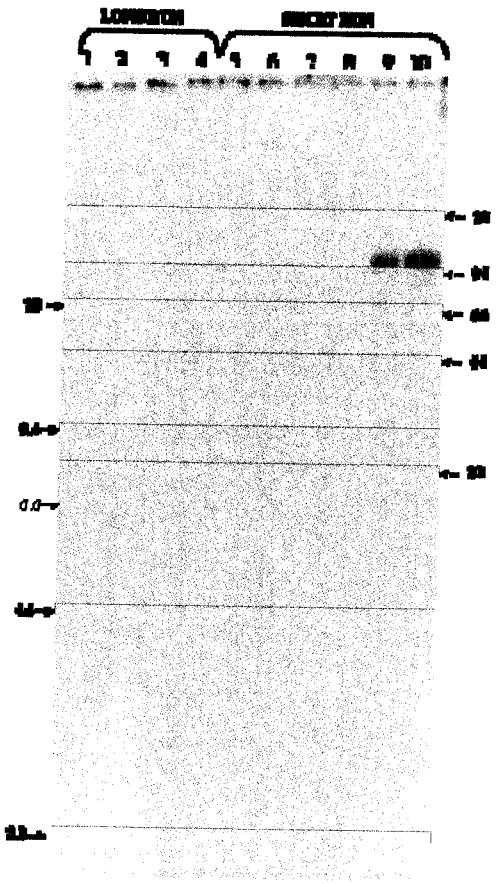


Figure 15. Southern blot analysis of MON 89034: PV-ZMIR245 backbone sequence

The blot was examined with ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

iv) Confirmation of the Absence of T-DNA II

- **Southern blot analysis confirming the absence of the *nptII* coding sequence**

Southern blot analysis was used to confirm that the *nptII* sequence necessary for transformation is no longer present in MON 89034. This is important to ensure that only desired proteins are produced in MON 89034.

The results of this analysis are presented in **Figure 16**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *nptII* coding sequence probe showed no detectable hybridisation bands, as expected for the negative control.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of ~10 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) of *Nco* I (lanes 3 and 7) produced no detectable hybridisation bands, indicating that MON 89034 contains no *nptII*-derived elements.

- **Southern blot analysis confirming absence of the overall T-DNA II region**

This analysis confirms the absence of the *nptII* coding sequence and demonstrates the absence of any additional T-DNA II sequences except for those elements that are shared with the T-DNA I expression cassette. This analysis confirms that the F_1 plants that contained T-DNA II elements were segregated out and that the plants selected for further development were containing only T-DNA I (please also refer to **Section 2.3.a.**).

The results of this analysis are presented in **Figure 17**. Conventional maize control DNA digested with *Ssp* I (lanes 2 and 6) and *Bst*E II (lanes 4 and 8) examined with overlapping probes spanning T-DNA II showed no hybridisation bands.

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Bst*E II (lanes 9 and 10) produced the two expected size bands at ~7.8 and ~2 kb. The overlapping probes spanning T-DNA II contain the *35S* promoter, *nos* 3' end, and Left Border sequences which are contained on T-DNA I. Therefore, the T-DNA II probe is expected to hybridise to bands derived from T-DNA I.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced two bands of ~8.2 and ~7.4 kb. The presence and size of these bands is consistent with the 8.2 and 7.4 kb bands detected during the analyses of the *e35S* promoter/*Cab* leader region (**Figure 7**, lanes 1 and 5) and *nos* 3' end sequences (**Figure 14**, lanes 1 and 5).

MON 89034 DNA digested with *Bst*E II (lanes 3 and 7) produced two bands of ~4.2 and ~1.7 kb. The ~4.2 kb band is consistent with the >2.8 kb expected band for sequence from T-DNA I digested with *Bst*E II (**Figure 3**), and the ~1.7 kb band is consistent with the T-DNA I specific band observed in **Figure 14**, lanes 3 and 7. No unexpected bands were detected, indicating that MON 89034 contains no additional elements other than those which are common with T-DNA I.

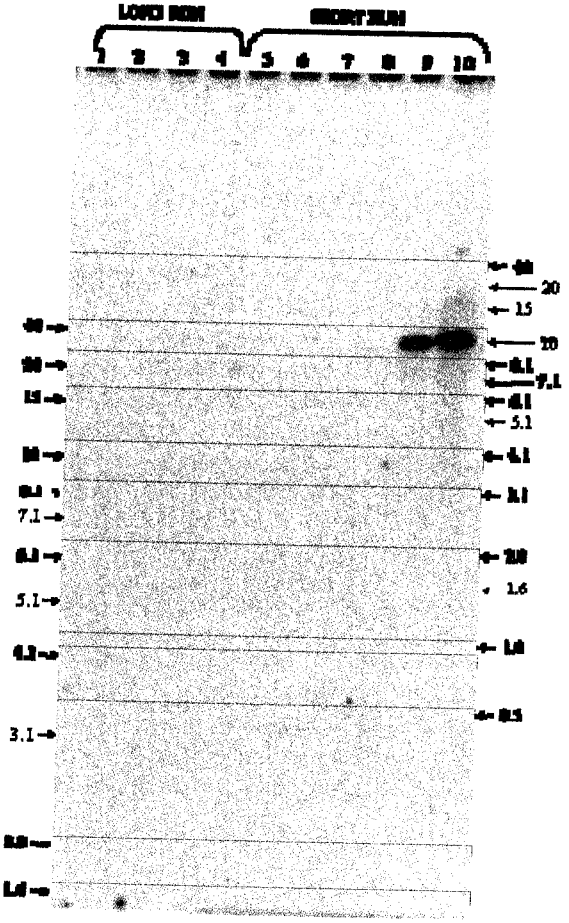


Figure 16. Southern blot analysis of MON 89034: *npt II* coding sequence
The blot was examined with a ^{32}P-labeled probe that spanned the *npt II* coding sequence (probe 12, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional maize (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional maize (*Nco I*)
9: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

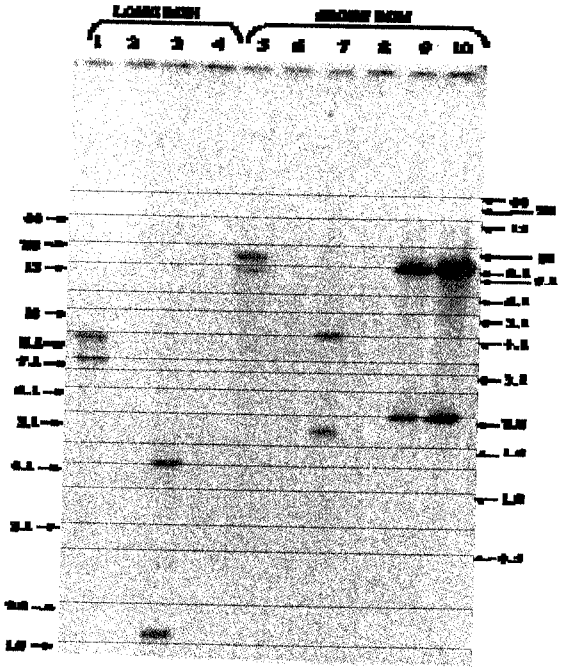


Figure 17. Southern blot analysis of MON 89034: T-DNA II
The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional maize (*Ssp* I)
3: MON 89034 (*Bst*E II)
4: Conventional maize (*Bst*E II)
5: MON 89034 (*Ssp* I)
6: Conventional maize (*Ssp* I)
7: MON 89034 (*Bst*E *II*)
8: Conventional maize (*Bst*E *II)*
9: Conventional maize spiked with PV-ZMIR245 (*Bst*E II) [0.5 copy]
10: Conventional maize spiked with PV-ZMIR245 (*Bst*E II) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

v) Organisation of the Insert Genetic Elements of MON 89034

The reported organisation of the elements within the insert in MON 89034 was confirmed using PCR analysis by amplifying seven overlapping regions of DNA that span the entire length of the insert. The location of the PCR products generated in relation to the insert, as well as the results of the PCR analyses, are shown in **Figure 18**. The control reactions containing no template DNA (lanes 2, 5, 8, 12, 16, 20, and 23) and the conventional maize control reactions in lanes 3, 6, 9, 13, 17, and 21 did not generate PCR products with any of the primer sets, as expected. The conventional maize control reaction in lane 24 produced a product of equal size to that of MON 89034 (lane 25) because both primer sequences are located in the flanking sequence adjacent to the 3' end of the insert in MON 89034. Additionally, the products generated using plasmid PV-ZMIR245 DNA as a template (lanes 11 and 15) appear overloaded in comparison to the MON 89034 genomic DNA samples which likely contributes to the intensity of the non-specific products observed in these lanes.

MON 89034 DNA generated the expected size PCR products of approximately 2.5 kb for Product A (lane 4); approximately 3.3 kb for Product B (lane 7); approximately 2.6 kb for Product C (lane 10); approximately 2.6 kb for Product D (lane 14); approximately 3.2 kb for Product E (lane 18), approximately 1.1 kb for Product F (lane 22) and approximately 0.8 kb for Product G (lane 25). The generation of the predicted size PCR products from MON 89034 establishes that the arrangement of linkage of elements in the insert are the same as those in plasmid PV-ZMIR 245 and that the elements within each gene cassette are arranged as depicted in the schematic of the insert in **Figure 3**.

PCR products generated from MON 89034 were subjected to DNA sequencing to further confirm the organisation of the elements within the insert. The consensus sequence representing the insert in MON 89034, including the genomic DNA flanking the ends of the insert, is shown in **Figure 1 in Appendix I: Confidential Attachment** and described in **Table 3.** This consensus sequence was generated by compiling numerous sequencing reactions performed on the PCR products which spanned the length of the insert and the 5' and 3' junctions with the flanking maize genomic DNA.

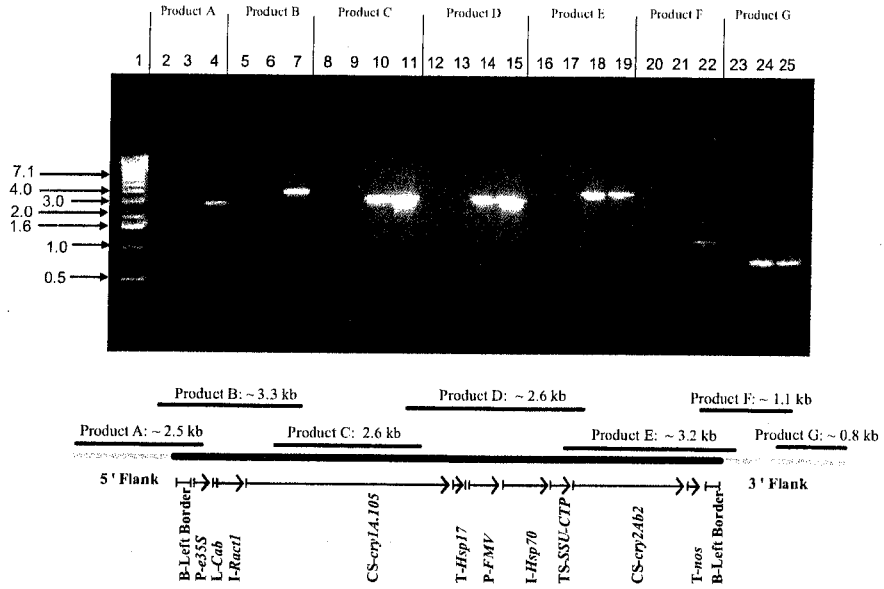
The insert is 9317 bases long and matches the sequence of PV-ZMIR245 as stated in the following:

1) The 5' end of the insert, beginning at base 2061 and ending at base 2384 in **Figure 1 in Appendix I: Confidential Attachment** matches the sequence of PV-ZMIR245 between bases 14696 and 14373 in **Figure 5**. It is possible that a crossover event occurred during the transformation resulting in a left border and modified e35S promoter at the 5' end of the insert.

2) The remainder of the insert, beginning at base 2385 and ending at base 11377 in **Figure 1 in Appendix I: Confidential Attachment**, matches PV-ZMIR245 between bases 2083 and 11075 in **Figure 5**.

In addition to the insert sequence, 2060 base pairs of maize genomic DNA flanking the 5' end of the insert and 905 base pairs of maize genomic DNA flanking the 3' end of the insert are reported in **Figure 1 in Appendix I: Confidential Attachment.**

Therefore, the results confirmed that the sequence of the DNA insert in MON 89034 matched the designed, corresponding sequences in PV-ZMIR245 with one exception. The *e35S* promoter that regulates expression of the *cry1A.105* gene has been modified and that the Right Border sequence present in PV-ZMIR245 was replaced by a Left Border sequence in MON 89034. This molecular rearrangement can be explained by a recombination event, which occurred either prior to or during the process of T-DNA transfer to the plants cell, between the DNA sequences near the *35S* promoters in T-DNA I and T-DNA II (**Figure 19**). Due to this recombination event, the reconstituted *e35S* promoter in MON 89034 (referred to as modified *e35S* or $e35S^{89}$) no longer has the duplicated enhancer elements compared to the original *e35S* promoter in PV-ZMIR245. Despite the deletion of the enhancer elements, the modified *e35S* promoter in MON 89034 regulates Cry1A.105 protein expression to provide sufficiently high levels for efficacious control of target insect pests.



→ Symbol denotes sizes, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

Figure 18. Overlapping PCR analysis across the insert in MON 89034

PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 89034 were performed on MON 89034 genomic DNA extracted from grain (Lanes 4, 7, 10, 14, 18, 22, and 25). Lanes 3, 6, 9, 13, 17, 21, and 24 contain reactions with conventional maize control DNA while lanes 2, 5, 8, 12, 16, 20, and 23 are reactions containing no template DNA. Lanes 11, 15, and 19 contain reactions with PV-ZMIR245 control DNA. Lane 1 contains Invitrogen 1 kb DNA ladder. Lanes are marked to show which product has been loaded and is visualised on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 89034 that appears at the bottom of the figure. Ten to twenty-five microliters of each of the PCR products were loaded on the gel. This figure is representative of the data generated in the study; however the specific bands from this gel were not excised and sequenced.

Lane
1: Invitrogen 1 kb DNA ladder
2: No template DNA control
3: Conventional maize control DNA
4: MON 89034 genomic DNA
5: No template DNA control
6: Conventional maize control DNA
7: MON 89034 genomic DNA
8: No template DNA control
9: Conventional maize control DNA
10: MON 89034 genomic DNA
11: PV-ZMIR245 plasmid
12: No template DNA control
13: Conventional maize control DNA
14: MON 89034 genomic DNA
15: PV-ZMIR245 plasmid
16: No template DNA control
17: Conventional maize control DNA
18: MON 89034 genomic DNA
19: PV-ZMIR245 plasmid
20: No template DNA control
21: Conventional maize control DNA
22: MON 89034 genomic DNA
23: No template DNA control
24: Conventional maize control DNA
25: MON 89034 genomic DNA

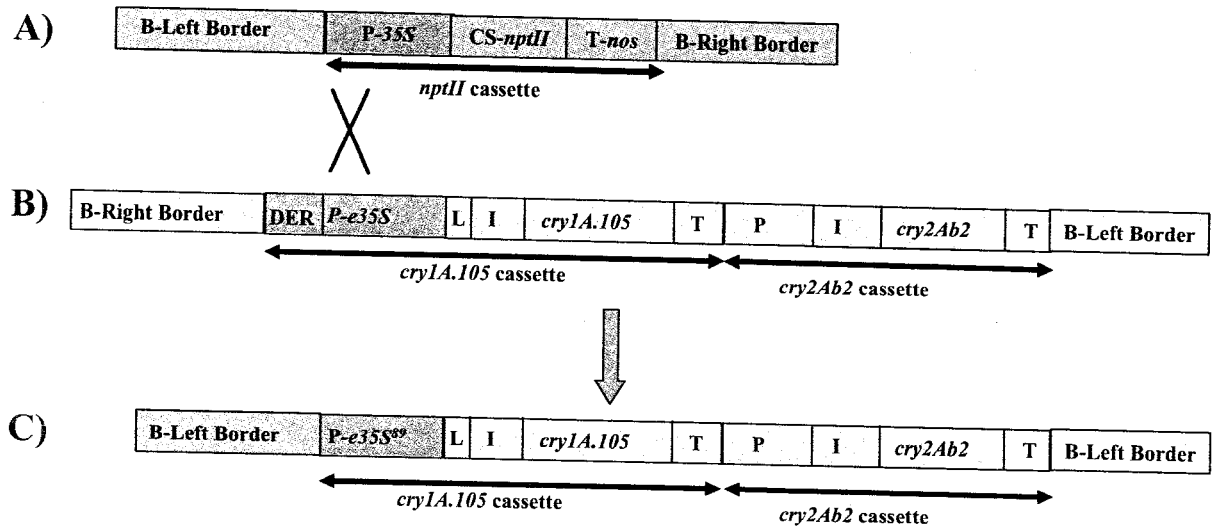


Figure 19. Description of the recombination process that explains the modified 5'end of the insert

A) Illustration of the T-DNA II of plasmid PV-ZMIR245.

B) Illustration of the T-DNA I of plasmid PV-ZMIR245.

C) Illustration of the modified T-DNA I in MON 89034.

Abbreviations and symbols: DER = duplicated enhancer region; L = leader sequence; I = intron sequence; P = promoter; T = termination sequence.

The diagram illustrates a recombination event, which likely occurred prior to or during the process of T-DNA transfer to the plant cells, between the DNA sequences near the *35S* promoters in T-DNA I and T-DNA II. Due to this recombination event, the reconstituted *e35S* promoter in MON 89034 (referred to as modified *e35S* or $e35S^{89}$) no longer has the duplicated enhancer elements (DER) compared to the original *e35S* promoter in PV-ZMIR245. Despite the deletion of the enhance elements, the Cry1A.105 protein expression levels in MON 89034 are still sufficiently high under the regulation of the modified *e35S* promoter to deliver the required efficacy against target insect pests.

Table 3. Summary of Genetic Elements in MON 89034

Genetic Element	Location in Sequence	Function (Reference)
Sequence flanking the 5' end of the insert	1-2060	Maize genomic DNA.
B[1]-Left Border[1]	2061-2299	239bp DNA region from the B-left Border region remaining after integration.
Intervening Sequence	2300-2349	Sequence used in DNA cloning.
P-*e35S*[89]	2350-2651	Modified e35s promoter and 9bp leader resulting from a recombination between the P-*e35s* and P-*35s* promoters.
Intervening Sequence	2652-2677	Sequence used in DNA cloning.
L[2]-*Cab*	2678-2738	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al., 1985).
Intervening Sequence	2739-2754	Sequence used in DNA cloning.
I[3]-*Ract1*	2755-3234	Intron from the rice actin gene (McElroy et al., 1991).
Intervening Sequence	3235-3243	Sequence used in DNA cloning.
CS[4]-*cry1A.105*[5]	3244-6777	Coding sequence for the *Bacillus thuringiensis* Cry1A.105 protein (Monsanto unpublished data).
Intervening Sequence	6778-6808	Sequence used in DNA cloning.
T[6]-*Hsp17*	6809-7018	3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation (McElwain and Spiker, 1989).
Intervening Sequence	7019-7085	Sequence used in DNA cloning.

[1] B – border region
[2] L - leader
[3] I - intron
[4] CS – coding sequence
[5] Coding sequence of the *cry1A.105* including stop codon
[6] T – transcript termination sequence

Table 3 (cont.) Summary of Genetic Elements in MON 89034

Genetic Element	Location in Sequence	Function (Reference)
P[1]-*FMV*	7086-7649	Figwort Mosaic Virus 35S promoter (Rogers, 2000).
Intervening Sequence	7650-7671	Sequence used in DNA cloning.
I-*Hsp70*	7672-8475	The first intron from the maize heat shock protein 70 gene (Brown and Santino, 1995).
Intervening Sequence	8476-8491	Sequence used in DNA cloning.
TS*[2]*-SSU-CTP	8492-8892	DNA region containing the targeting sequence for the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit and the first intron (Matsuoka et al., 1987).
CS-*cry2Ab2*	8893-10800	Coding sequence for a Cry2Ab2 protein from *Bacillus thuringiensis* (Widner and Whitely, 1989; Donovan, 1991). This coding sequence uses a modified codon usage.
Intervening Sequence	10801-10826	Sequence used in DNA cloning.
T-*nos*	10827-11079	3' termination sequence of the nopaline synthase (*nos*) coding sequence from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983).
Intervening Sequence	11080-11146	Sequence used in DNA cloning.
B-Left Border[2]	11147-11377	230bp DNA region from the B-Left Border region remaining after integration.
Sequence flanking the 3′ end of the insert	11378-12282	Maize genomic DNA.

[1] P – promoter
[2] TS – targeting sequence

vi) Alignment of the MON 89034 Insert DNA Sequence to the PV-ZMIR245 Transformation Vector DNA Sequence

The reported sequence of the insert in MON 89034 was aligned to the sequence of the transformation vector PV-ZMIR245 using the BestFit function in SeqLab[1]. Previously it was determined that the insert in MON 89034 matches the sequence of PV-ZMIR245 (please refer to **Section 2.3.c.v.**). The 5' end of the insert, beginning at base 2061 and ending at base 2384 matches the sequence of PV-ZMIR245 between bases 14696 and 14373 (**Figure 2 in Appendix I: Confidential Attachment**). The remainder of the insert, beginning at base 2385 and ending at base 11377, matches PV-ZMIR245 between bases 2083 and 11075 (**Figure 3 in Appendix I: Confidential Attachment**). The sequence of the insert in MON 89034 is 100% identical to the corresponding sequences in PV-ZMIR245.

These results indicate that the P-*e35S* promoter that regulates expression of the *cry1A.105* gene has been modified and that the Right Border sequence present in PV-ZMIR245 was replaced by a Left Border sequence. It is likely that this modification is the result of a crossover recombination event that occurred prior to the DNA being inserted into the genome between *35S* promoters in T-DNA I and T-DNA II (**Figure 19**). Therefore a modified *e35S* promoter regulates expression of the *cry1A.105* gene in MON 89034. The remaining MON 89034 insert sequence was not altered during the transformation process.

vii) Bioinformatics evaluation of the DNA Sequences Flanking the 5' and 3' Junctions of the Inserted DNA in MON 89034: Assessment of Putative Polypeptides

Bioinformatics analyses were performed on putative polypeptides deduced from DNA sequences spanning the 5' and 3' inserted DNA-genomic DNA junctions of MON 89034 to assess the potential for similarity towards allergens, toxins, or other bioactive proteins.

DNA sequences flanking the 5' and 3' junctions of the insertion site in MON 89034 were translated from stop codon to stop codon in all possible reading frames. Polypeptide sequence from each reading frame was then inspected to confirm that the sequence was both encoded by DNA spanning the inserted DNA-genomic DNA junctions and was greater than or equal to eight amino acids in length. At the 5' flank, five deduced putative polypeptides spanned the genomic DNA-inserted DNA junction, while at the 3' flank, five putative polypeptides spanned the inserted DNA-genomic DNA junction.

Hypothetical polypeptides from each reading frame were compared to allergen (AD6), toxin (TOXIN5), and public domain (ALLPEPTIDES) database sequences using the FASTA sequence alignment tool; used to assess structural relatedness between the query sequences and any protein sequences in the AD6, TOXIN5 and ALLPEPTIDES databases. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity, and the *E* score. The *E* score reflects the degree of similarity between a pair of sequences,

[1] SeqLab, Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA.

and it can be used to evaluate the significance of an alignment. The calculated *E*-score depends on the overall length of joined (gapped) local sequence alignments, the quality (percent identity, similarity) of the overlap, and the size of the database (Pearson and Lipman, 1988; Baxevanis and Ouellette, 1998). For a pair of sequences, a very small *E* score may indicate a structurally relevant similarity. Conversely, large *E* scores are typically associated with alignments that do not represent a biologically relevant structural similarity. An *E* score of 1×10^{-5} was further analysed to determine if such an alignment represented a bona fide sequence homology.

In addition to structural similarity, each putative polypeptide was screened for short polypeptide (eight amino acid) matches using a pair-wise comparison algorithm. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain immunologically significant epitopes and therefore it is recommended that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides (Codex, 2003). In these analyses, eight linearly contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope.

No biologically relevant structural similarity to allergens, toxins, or bioactive proteins was observed for any of the hypothetical polypeptides from the alignment searches. The results of these bioinformatic analyses demonstrate that even in the highly unlikely event that any of the junction polypeptides were translated; they would not share a sufficient degree of similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other health implications.

d) Segregation and Stability of the Inserted DNA

i) Description of the MON 89034 breeding process

As described in **Section 2.3.a.**, a proprietary inbred line, LH172 was utilised as the initial recipient of the transgenes in MON 89034. Freshly isolated immature maize embryos were transformed with the plasmid PV-ZMIR245 containing the 2T-DNA system.

Immature embryos were grown on a selection medium containing carbenicillin to eliminate *Agrobacterium*, and paromomycin to eliminate cells that were not transformed, so that only cells containing T-DNA II and/or T-DNA I + T-DNA II survived (i.e., plants containing the *nptII* expression cassette). The resulting transformed cells were then subcultured several times on a selection medium and regenerated into the R_0 plants according to the protocol described by Armstrong and Phillips (1988).

The R_0 plant was then cross-pollinated with the recurrent parent LH172 (same as transformed maize line) to produce the LH172 $BC0F_1$ generation. During this breeding step, the unlinked insertions of T-DNA I or T-DNA II were segregated. Only the plants which were positive in *cry1A.105* PCR assay and negative in NPTII TaqMan assay were selected, while plants containing the *nptII* cassette (T-DNA II) were eliminated from subsequent breeding. The selected LH172 $BC0F_1$ plants were self-pollinated and advanced to LH172 $BC0F_2$. The segregation in the LH172 $BC0F_2$

generation was examined using ELISA for Cry2Ab2 on leaf tissue. The selected LH172 $BC0F_2$ plants were self-pollinated to generate LH172 $BC0F_3$. The segregation in LH172 $BC0F_2$ generation was determined using ELISA for Cry1A.105 and GeneCheck® immunoassay for Cry2Ab2 protein on leaf tissue. The F_3 generation was fixed for the DNA insert and not expected to segregate further. The stability of the F_4 and F_5 generations was confirmed using GeneCheck® immunoassay for Cry2Ab2 on leaf tissue. Without exception, all χ^2 values indicated no significant differences between observed and expected genetic ratios across all tested generations.

The LH172 $BC0F_4$ generation crossed to trait integration corn materials (12 maize inbred lines) was used for commercial development. Generations LH172 $BC0F_6$ and LH172 $BC0F_7$ crossed to LH198 (hybrid seed) were used in all the other analyses (composition and expression analyses) and additional field trials.

Please also refer to **Figure 20**.

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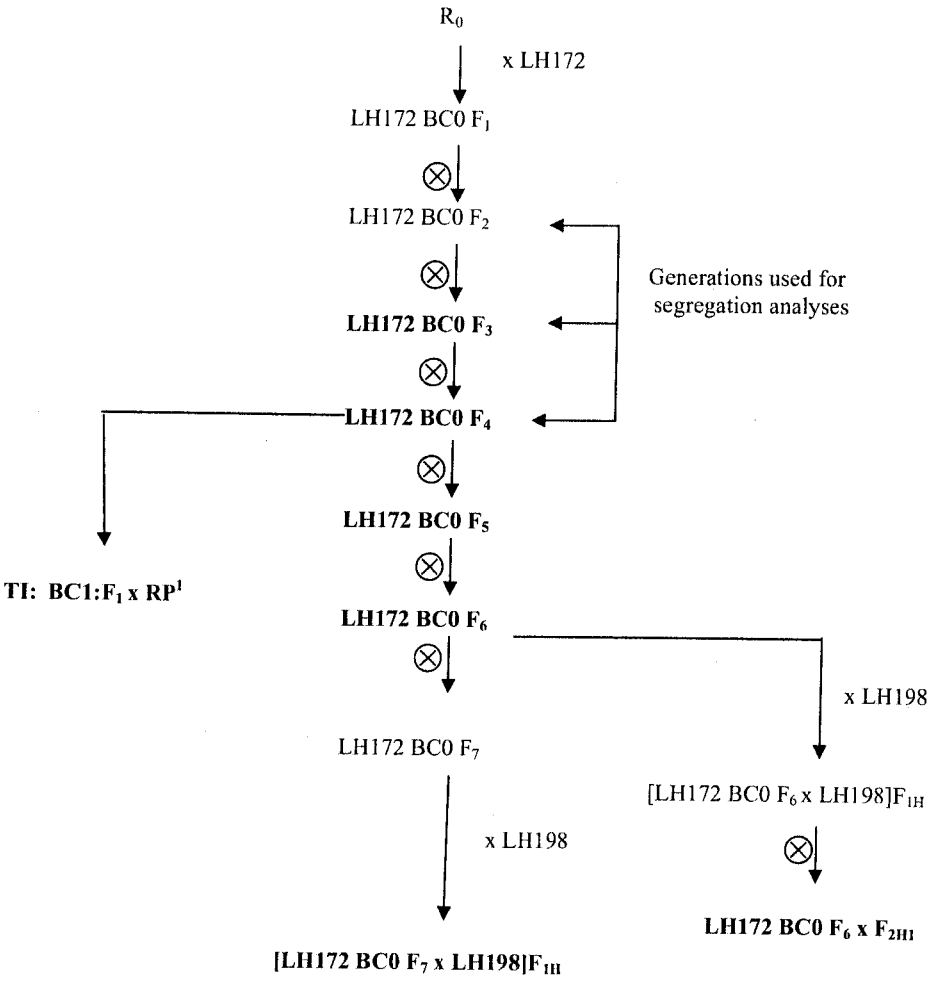

R_0 = transformed plant; F(#) = filial generation; ⊗ = self-pollination; BC(#) = backcross generation.; RP = recurrent parent; H = hybrid; TI = Trait Integration

Figure 20. Breeding history of MON 89034
The LH172 BC0 F_6 x F_{2H1} generation was used for all molecular analyses. Generations used for molecular stability analysis are shown in bold in the breeding tree.
[1] Recurrent parent (RP) that was used in the analyses is referred to as Conventional maize A.

ii) Inheritance of the Lepidopteran Protection Trait

Significance of the segregation pattern (positives/negatives) was assessed by Chi square analysis over four generations of MON 89034 to determine the heritability and stability of the trait (*cry1A.105* and the *cry2Ab2* genes, and Cry1A.105 and Cry2Ab2 proteins) (**Table 4**). The confirmation of the presence of the gene and stability of the trait was based on one of several assays: 1) ELISA to detect Cry2Ab2 protein; 2) ELISA to detect Cry1A.105 protein; 3) GeneCheck® immunoassays to detect Cry2Ab2 protein (Cry2A QuickStix Lateral Flow test strips, Envirologix Inc., Portland, MN); and 4) PCR assay to detect the presence of the *cry* genes.

As described earlier, MON 89034 was developed using a 2T-DNA vector transformation system that employed two separate T-DNAs. One T-DNA (T-DNA I) contained the *cry1A.105* and *cry2Ab2* genes while the other T-DNA (T-DNA II) contained the *nptII* gene. F_1 plants were generated in a LH172 germplasm by making crosses of the R_0 plant with the LH172 inbred. From the population of produced F_1 plants, selections were made for the absence of the *nptII* gene, and the plants were screened for copy number of the *cry1A.105* and *cry2Ab2* inserted cassettes using Southern blot analysis. Plants selected in the F_1 generation were either used to make $BC1F_1$ seed, or were self-pollinated to generate F_2 seed (**Figure 20**). The overall goal of the F_1 population of plants was to select single copy, marker-free plants. A final plant was selected from the F_1 generation, designated as event MON 89034, and progeny derived from this plant showed the expected patterns for genetic segregation (**Table 4**).

The Chi-square analysis is based on testing the observed segregation ratio of the Cry proteins to the ratio that is expected according to Mendelian principles as shown below:

Generation	Expected Ratio[a]	Comment
LH172 $BC0F_1$	n.a.	screened for copy number and absence of *nptII* (segregation data not shown)
LH172 $BC0F_2$	3:1	positive:negative (product of self pollination)
LH172 $BC0F_3$	1:0	positive:negative (homozygous plant selection)
LH172 $BC0F_4$	1:0	positive:negative (homozygous plant selection)
LH172 $BC1F_1$[b]	1:1	positive:negative (product of backcrossing)
LH172 $BC1F_2$[c]	3:1	positive:negative (product of self pollination)
LH172 $BC1F_2$[c]	3:1	positive:negative (product of self pollination)

[a] n.a. = not applicable.
[b] To confirm segregation, LH172 $BC0F_1$ plants were backcrossed to the recurrent parent (LH172) to produce this generation (not shown on the breeding tree, **Figure 20**).
[c] To confirm segregation, The LH172 $BC1F_1$ plants were selfed to produce two different plant populations of this generation (not shown on the breeding tree, **Figure 20**).

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The Chi-square test was computed as:

$$\chi^2 = \Sigma [(| o - e | - 0.5)^2 / e]$$

where o = observed frequency of the genotype, e = expected frequency of the genotype, and 0.5 = Yates correction factor for analysis with one degree of freedom (Little and Hills, 1978).

Results of the Chi-square test are summarised in **Table 4**. All χ^2 values indicated no significant differences between observed and expected genetic ratios across all tested generations of MON 89034. These results are consistent with molecular characterisation data indicating a single insertion site for the *cry1A.105* and *cry2Ab* expression cassettes (please refer to **Section 2.5.c.i.**).

Table 4. Segregation Analyses of MON 89034

Generation	Number of plants	Observed Positives [a]	Observed Negatives	Expected Positives [a]	Expected Negatives	Chi-Square	Probability
LH172 $BC0F_2$	11	7	4	8.25	2.75	0.2727	>0.05
LH172 $BC0F_3$	24	24	0	24	0	Fixed +	n.a.
LH172 $BC0F_4$	30	30	0	30	0	Fixed +	n.a.
LH172 $BC1F_1$	28	13	15	14	14	0.0357	>0.05
LH172 $BC1F_2$	24	20	4	18	6	0.5	>0.05
LH172 $BC1F_2$	24	17	7	18	6	0.0556	>0.05

[a] The confirmation of the trait was based on one of several assays: 1) ELISA to detect the Cry2Ab2 protein; 2) ELISA to detect the Cry1A.105 protein; 3) PCR assay to detect presence of the *cry* genes; and, 4) GeneCheck immunoassays to detect the Cry2Ab2 protein.

iii) Insert stability across generations of MON 89034

To demonstrate the stability of the MON 89034 insert during breeding, additional Southern blot analyses were performed using DNA samples obtained from multiple generations of the MON 89034 breeding history. For reference, the breeding history of MON 89034 is presented in **Figure 20**. The specific generations tested are indicated in the legends of **Figures 21, 22**, and **23**.

For these analyses, DNA samples were digested with the restriction enzyme *Ssp* I. *Ssp* I cleaves once within the inserted DNA and in both the 5' and 3' genomic flanking sequences of MON 89034. This produces two DNA fragments of ~8.2 and >4.3 kb (**Figure 3**). The ~8.2 kb fragment contains genomic DNA flanking the 5' end of the insert, Left Border sequence, modified *e35S* promoter sequence, *Cab* leader, *Ract1* intron, *cry1A.105* coding sequence, *Hsp17* 3' end sequence, *FMV* promoter, and a portion of the *Hsp70* intron. The >4.3 kb fragment contains the remaining portion of the *Hsp70* intron, *SSU-CTP* targeting sequence, *cry2Ab2* coding sequence, *nos* 3' end sequence, Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA (**Figure 3**).

Plasmid PV-ZMIR245 DNA was spiked into the conventional maize control DNA, digested with *Ssp* I, and loaded on the gel to serve as a positive hybridisation control. Individual Southern blots were examined with three-probe sets. The stability of the MON 89034 insert across generations was confirmed using overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18 – 23 in **Figure 5**). The absence of the *nptII* selectable marker and unique T-DNA II genetic elements not contained in T-DNA I was confirmed using overlapping probes spanning T-DNA II (probes 11, 12, and 13, **Figure 4**). The absence of plasmid PV-ZMIR245 backbone sequence across generations was confirmed using overlapping probes spanning the vector backbone of PV-ZMIR245 (probes 14 - 17, **Figure 4**). A second conventional maize control (referred to as conventional maize A) was used in these Southern blots to ensure that the genetic backgrounds of all the generations were accurately represented.

- **Stability of the T-DNA I insert across generations**

The results of this analysis are presented in **Figure 21**. Conventional maize control DNA digested with *Ssp* I (lane 7 and 9) and examined with overlapping T-DNA I probes spanning the entire inserted DNA sequence showed several faint hybridisation bands (**Figure 21**). This was expected because T-DNA I contain several genetic elements originally derived from maize. These endogenous bands were only detected in conventional maize or darker exposures and were seen previously when blots were probed with the overlapping T-DNA I probe (see **Figure 6**), the *Hsp 70* intron probe (**Figure 12**), and the *SSU-CTP/cry2Ab2* probe (**Figure 13**).

Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at ~10.4 and ~7.2 kb in addition to the expected endogenous bands. The migration of the ~10.4 kb fragment is slightly higher than indicated by the molecular marker band sizes in the lanes 10 and 11. The altered migration may be due to the difference in salt concentration between the maize DNA samples and the molecular marker (Sambrook and Russell, 2001).

DNA extracted from seven MON 89034 generations digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) each produced two bands of ~8.2 and ~7.4 kb in addition to the expected endogenous hybridisation. The ~8.2 kb band is the expected size for the 5' border fragment and the ~7.4 kb band is consistent with the expected band size of >4.3 kb for the 3' border fragment. These bands are consistent with the bands detected in **Figure 6** (lanes 3 and 7).

Two faint bands of ~15.6 and ~12 kb were observed in lanes 3 and 4. These bands likely are the result of partial digestion because they are not seen in prior or subsequent generations that were produced by self pollination. In support of this conclusion the presence of the ~15.6 kb band is consistent with the lack of digestion at the internal *Ssp* I site. This band would be expected as a combination of the 7.4 and 8.2 kb bands observed for *Ssp* I digestion. The ~12 kb band is consistent with the partial digestion of the *Ssp* I site in the 5' flanking genomic sequence which would result in an ~11.8 kb band.

No additional unexpected bands were detected, indicating that the single copy of T-DNA I in MON 89034 is stable in the selected generations.

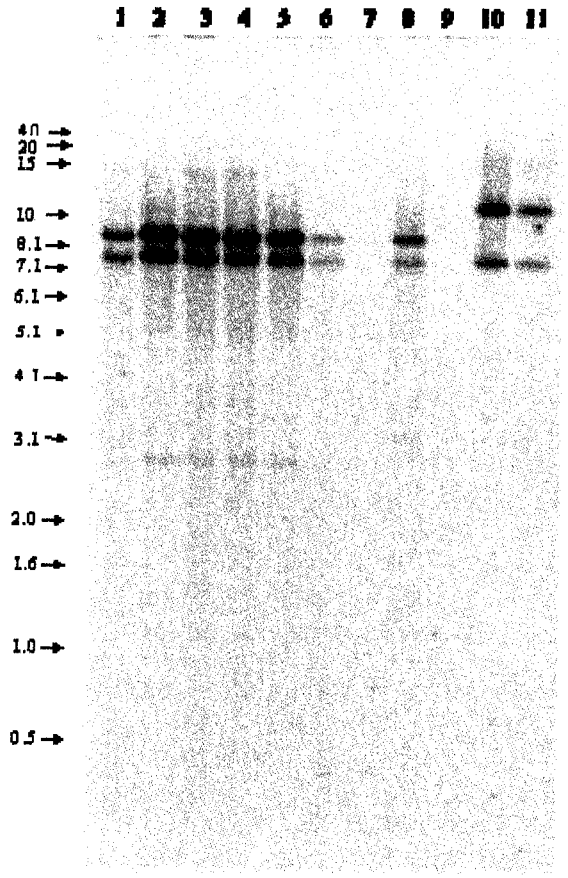

Figure 21. Insert stability of MON 89034: T-DNA I

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 - 23, **Figure 5**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0F_6 x F_{2H1}, *Ssp* I)
2: MON 89034 (LH172 BC0 F_3, *Ssp* I)
3: MON 89034 (LH172 BC0 F_4, *Ssp* I)
4: MON 89034 (LH172 BC0 F_5, *Ssp* I)
5: MON 89034 (LH172 BC0 F_6, *Ssp* I)
6: MON 89034 ([LH172 BC0 F_7 x LH198]F_{1H}, *Ssp* I)
7: Conventional maize (*Ssp* I)
8: MON 89034 (TI: BC1: F_1 x RP, *Ssp* I)
9: Conventional maize A[1] (*Ssp* I)
10: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
11: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

[1] Monsanto proprietary conventional maize hybrid.

- **Confirmation of the absence of T-DNA II in multiple generations of MON 89034**

The results of this analysis are presented in **Figure 22**. Conventional maize control DNA digested with *Ssp* I (lane 7 and 9) and examined with three overlapping probes spanning T-DNA II showed no detectable hybridisation bands. Conventional maize control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at ~10 and ~7.2 kb. The migration of the ~10 kb fragment is slightly higher than indicated by the molecular marker band sizes in the lanes 10 and 11. The altered migration may be due to the difference in salt concentration between the maize DNA samples and the molecular marker (Sambrook and Russell, 2001). The overlapping probes spanning T-DNA II contains the *35S* promoter, *nos* 3' end, and the Left Border which are contained on T-DNA I. Therefore, the T-DNA II probe is expected to hybridise to fragments derived from T-DNA I.

DNA extracted from seven generations of MON 89034 digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) each produced two bands of ~8.2 and ~7.4 kb which are consistent with those observed with the overlapping T DNA I probes. The ~ 8.2 kb band is the expected size for the 5' border fragment and the ~7.4 kb band is consistent with the expected band size of > 4.3 kb for the 3' border fragment.

On longer exposures, two faint bands of ~15.6 and ~12 kb were observed in lanes 3 and 4. These bands are consistent with the bands observed in **Figure 21** (lanes 3 and 4) and are likely the result of partial digestion because they are not seen in prior generations produced by self-pollination. The presence of the 15.6 kb band is consistent with the possibility that internal *Ssp* I site was not digested and a combination of the 7.4 and 8.2 kb bands produced the 15.6 kb band. The 12.0 kb band is consistent with the partial digestion of the *Ssp* I site in the 5' flanking genomic sequence which would result in an approximately 11.8 kb band (sequence data not reported).

No additional bands were detected, indicating that the seven generations tested do not contain any additional T-DNA II elements other than those which are common to T-DNA I and therefore the T-DNA II elements were segregated out as expected.

- **Confirmation of the absence of plasmid PV-ZMIR245 backbone sequence in multiple generations of MON 89034**

The results of this analysis are presented in **Figure 23**. Conventional maize control DNA, digested with *Ssp* I (lanes 7 and 9), and examined with four overlapping probes spanning the PV-ZMIR245 backbone sequence showed no detectable hybridisation bands. Although difficult to observe in **Figure 23**, overexposures of Southern blots showed that conventional maize A control DNA, digested with *Ssp* I (lanes 7 and 9), produced two faint hybridisation bands at 6.0 and 3.5 kb. These are likely the result of endogenous hybridisation to maize genetic elements specific to this background. Conventional control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at ~10.4 and ~7.2 kb.

DNA samples extracted from the seven generation digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) produced no hybridisation bands. This confirmed that the backbone sequences from PV-ZMIR245 are not present in MON 89034.

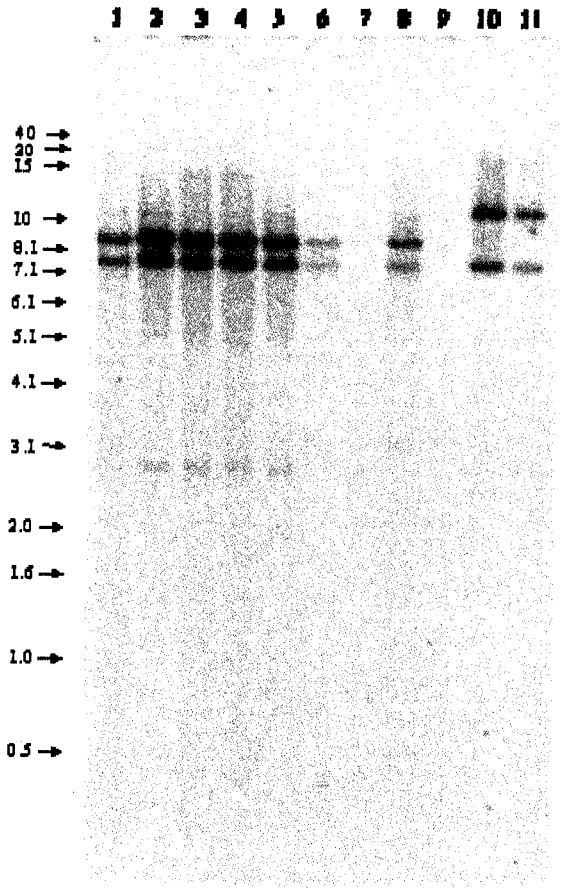

Figure 22. T-DNA II analysis of multiple generations of MON 89034: T-DNA II
The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0F_6 x F_{2H1}, *Ssp* I)
2: MON 89034 (LH172 BC0 F_3, *Ssp* I)
3: MON 89034 (LH172 BC0 F_4, *Ssp* I)
4: MON 89034 (LH172 BC0 F_5, *Ssp* I)
5: MON 89034 (LH172 BC0 F_6, *Ssp* I)
6: MON 89034 ([LH172 BC0 F_7 x LH198]F_{1H}, *Ssp* I)
7: Conventional maize (*Ssp* I)
8: MON 89034 (TI: BC1: F_1 x RP, *Ssp* I)
9: Conventional maize A[1] (*Ssp* I)
10: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
11: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

[1] Monsanto proprietary conventional maize hybrid

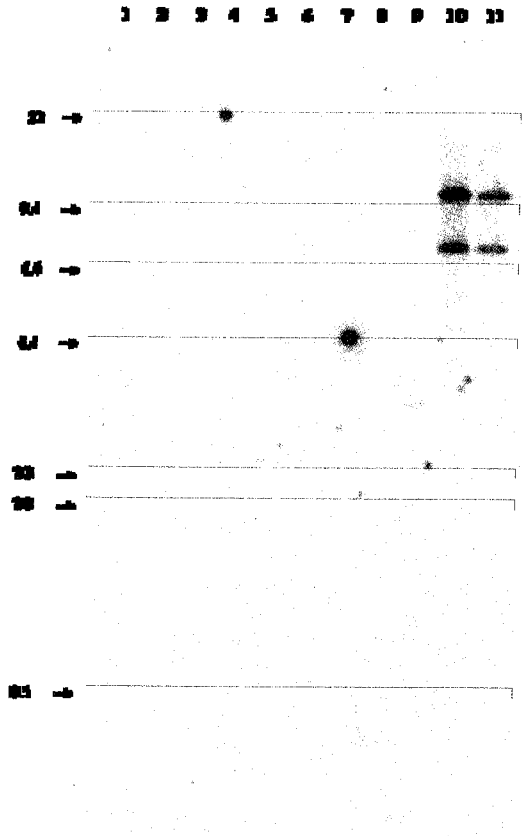

Figure 23. Backbone analysis of multiple generations of MON 89034: PV-ZMIR245 backbone sequence

The blot was examined with overlapping ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17, **Figure 4**). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0F_6 x F_{2H1}, *Ssp* I)
2: MON 89034 (LH172 BC0 F_3, *Ssp* I)
3: MON 89034 (LH172 BC0 F_4, *Ssp* I)
4: MON 89034 (LH172 BC0 F_5, *Ssp* I)
5: MON 89034 (LH172 BC0 F_6, *Ssp* I)
6: MON 89034 ([LH172 BC0 F_7 x LH198]F_{1H}, *Ssp* I)
7: Conventional maize (*Ssp* I)
8: MON 89034 (TI: BC1: F_1 x RP, *Ssp* I)
9: Conventional maize A[1] (*Ssp* I)
10: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
11: Conventional maize spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

[1] Monsanto proprietary conventional maize hybrid

2.4 Antibiotic Resistance Marker Genes

No genes that encode resistance to an antibiotic were inserted into the genome of MON 89034. Molecular characterisation data presented in **Section 2.3.c.iv.** confirms the absence of the *aad* and *nptII* genes encoding antibiotic resistance markers that were used in cloning and transformation processes.

2.5 Characterisation of Novel Protein

a) Description of the proteins and their function

MON 89034 produces two different Bt proteins, Cry1A.105 and Cry2Ab2, which are both highly efficacious against a variety of lepidopteran insect pests. The following sections describe the Cry1A.105 and Cry2Ab2 proteins and their function, including a characterisation of the introduced proteins.

Expression of proteins *in planta* is usually too low to allow purification of sufficient quantities for use in safety assessment studies. Therefore, it is necessary to produce the proteins in high-expressing recombinant host systems (such as bacteria) in order to obtain larger quantities of the protein(s) of interest. Proteins produced by the bacterial systems are engineered to match the amino acid sequences of their counterparts expressed in the biotechnology-derived plants. Thus, physicochemical and functional equivalence of plant- and bacteria-produced proteins need to be examined as part of the protein characterisation and safety assessment.

The bacteria derived Cry1A.105 and Cry2Ab2 proteins were produced in *Escherichia coli* and subsequently purified. Small quantities of the Cry1A.105 and Cry2Ab2 proteins were purified from the grain of MON 89034. The identities of the MON 89034-derived proteins were confirmed and the physicochemical properties and functional activities were compared to those of the bacteria-produced protein standards. The characteristics that were analysed to establish the equivalence between the recombinant *E. coli*- and the MON 89034-produced Cry1A.105 and Cry2Ab2 protein included:

1. SDS-PAGE analysis to estimate molecular weight;
2. Western blot analysis to demonstrate identity and immunoreactivity;
3. Confirmation of intactness of N-terminus of the protein with an antibody specific to the N-terminal peptide (for Cry1A.105 protein);
4. N-terminal sequencing by Edman degradation chemistry (for Cry2Ab2 protein);
5. Protein identification by tryptic peptide mapping using MALDI-TOF MS analysis;
6. Glycosylation analysis to examine the presence of absence of covalently linked carbohydrates; and
7. Insect activity bioassay to assess functional equivalence.

i) Identity and function of the Cry1A.105 protein

Cry1A.105 is a full-length, insecticidal protein consisting of 1177 amino acids with a molecular weight (MW) of 133 kDa. It is a chimeric protein that consists of domains I and II from Cry1Ab or Cry1Ac[1], substantially domain III from Cry1F, and the C-terminal domain from Cry1Ac (**Figure 24**). Cry1Ac, Cry1Ab and Cry1F are all well known and well characterised insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*). Cry1A.105 was designed using domain exchange strategy to achieve high levels of activity against target lepidopteran insect pests. The overall amino acid sequence identity of Cry1A.105 to Cry1Ac, Cry1Ab, and Cry1F is 93.6%, 90.0%, and 76.7%, respectively (**Table 5**). According to an accepted phylogram (Crickmore et al., 1998) for Bt crystal proteins, Cry1A.105 is found in the same cluster as Cry1Ac and also is related to Cry1Ab protein (Crickmore, 2004) (see also **Section 2.5.c.i.** and **Figure 38**).

Domain exchange is a well known naturally occurring mechanism that increases Bt protein diversities (De Maagd et al., 2001; Masson 2002; De Maagd et al., 2003). Domain exchange strategies with modern molecular biological tools have been used to switch the functional domains of Bt Cry1 proteins to develop commercial microbial biopesticides with improved specificity to lepidopteran insect pests. Microbial pesticides that contain a Cry1Ac/Cry1F chimeric protein have been used for control of lepidopteran pests since 1997 (Baum, 1998; Baum et al., 1999), and a biotechnology-derived cotton expressing a chimeric protein consisting of domains or sequences from Cry1F, Cry1C, and Cry1Ab has been commercialised (Gao et al., 2006).

The general mechanism of insecticidal activity of Cry proteins is well understood (Gill et al., 1992; Schnepf et al., 1998). Cry proteins are comprised of several functional domains that have highly conserved regions among the classes. For example, the amino acid sequence of Cry1A proteins is highly conserved in domains I, II and III. These functional domains have been shown to determine the activity and specificity of the Cry proteins. Domain I is involved in membrane insertion and pore formation, and domain II is involved in specific receptor recognition and binding as shown by mutagenesis studies. Domain III plays a role in receptor binding. The combination of domains I and II has been shown to determine insect specificity as well as specificity (De Maagd et al., 2001). The C-terminal domain is implicated in crystal formation which does not directly contribute to the insecticidal activity (De Maagd et al., 2001). The C-terminal domain is cleaved upon entry into the insect midgut or by certain proteases *in vitro*. Only insects with specific receptors are affected and no toxicity is observed in species that lack these receptors (Crickmore et al., 1998; De Maagd et al., 2001).

As with other Cry1A proteins, Cry1A.105 is active against major lepidopteran insect pests. The spectrum of activity includes corn borers from the genera *Ostrinia* and *Diatraea* (such as European corn borer, Asian corn borer, sugarcane borer, southwestern corn borer), armyworms (*Spodoptera* spp. including fall armyworm), earworms (*Helicoverpa* spp. including corn earworm), and cutworms (e.g., *Agrotis ipsilon*, black cutworm).

[1] Cry1Ab and Cry1Ac shares 100% amino acid sequence identity in domains I and II.

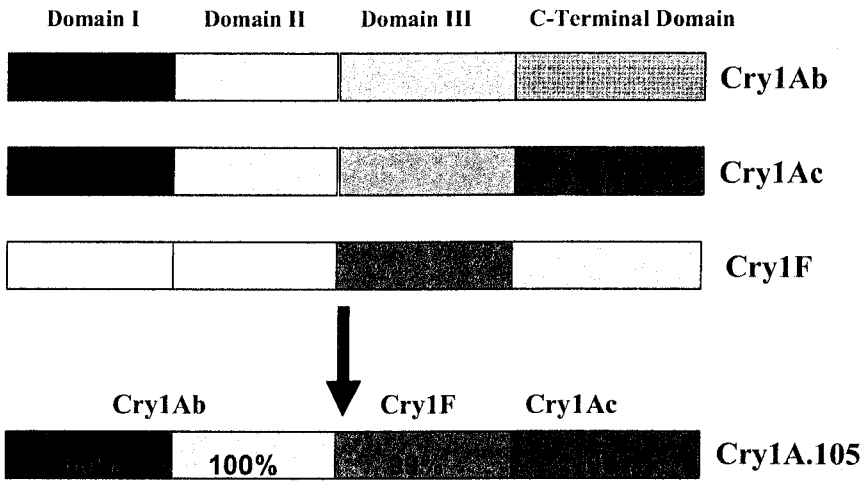

Figure 24. Schematic representation of the origin of Cry1A.105 protein domains. Different colour and patterns are used to differentiate the origin of domains. For simplicity, the lengths of domains in this illustration are not in proportion to the lengths of amino acid sequence of the respective domains.

Table 5. Amino acid sequence identity between Cry1A.105 and Cry1Ac, Cry1Ab, and Cry1F proteins

Domain	Amino acid identity to Cry1A.105 (%)		
	Cry1Ac	Cry1Ab	Cry1F
I	100	100	57
II	100	100	37
III	57	46	99
C-terminal	100	92	93
Overall	93.6	90	76.7

ii) Characterisation of the Cry1A.105 protein

The Cry1A.105 protein was purified from the grain of MON 89034 as well as from a fermentation of a recombinant *E. coli* strain. A panel of analytical tests was used to identify, characterise and compare the MON 89034-produced and the *E. coli*-produced Cry1A.105 proteins including: (1) SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) to examine molecular weight equivalence, (2) western blot analysis to demonstrate immunoreactivity and identity, (3) confirmation of N-terminus intactness with an antibody specific to the N-terminal peptide, (4) protein identification by tryptic peptide mapping by MALDI-TOF MS (matrix assisted laser desorption / ionisation time-of-flight mass spectrometry), (5) glycosylation analysis to examine the presence or absence of covalently linked carbohydrates; and (6) function activity in an insect bioassay to assess functional equivalence.

- **Cry1A.105 protein molecular weight equivalence**

The equivalence in molecular weight of the purified MON 89034- and the *E. coli*-produced Cry1A.105 protein was demonstrated using SDS-PAGE. The full-length MON 89034-produced Cry1A.105 protein migrated to a similar position compared to that of the *E. coli*-produced protein standard which was analysed concurrently (**Figure 25**).

Based on the comparable electrophoretic mobility, the MON 89034- and *E. coli*-produced Cry1A.105 protein were determined to have equivalent molecular weights.

- **Cry1A.105 protein immunoreactivity**

Western blot analysis using a polyclonal anti-Cry1A.105 antibody was conducted to determine the relative immunoreactivity of the purified MON 89034-produced Cry1A.105 protein and the *E. coli*-produced Cry1A.105 reference standard. The results demonstrated that the anti-Cry1A.105 antibody recognised the full-length MON 89034-produced Cry1A.105 protein that migrated similarly to the full-length *E. coli*-produced reference Cry1A.105 protein (**Figure 26**). As expected, the immunoreactive signal increased with increasing levels of loading for both MON 89034- and *E. coli*-produced proteins. The immunoreactive bands with lower molecular weight were present in both protein samples. These bands most likely represent proteolytic fragments of the Cry1A.105 protein. It is common to observe such proteolytic fragments of Cry1 proteins due to the cleavage by proteases *in vivo* or *in vitro*. A faint immunoreactive band with molecular weight above 250 kDa was observed in the samples of the MON 89034-produced and *E. coli*-produced Cry1A.105 protein. This band most likely represents the aggregate of the Cry1A.105 protein.

The western blot analysis confirmed the identity of the MON 89034-produced Cry1A.105 protein and demonstrated that the MON 89034- and *E. coli*-produced Cry1A.105 protein had equivalent immunoreactivity with the anti-Cry1A.105 antibody.

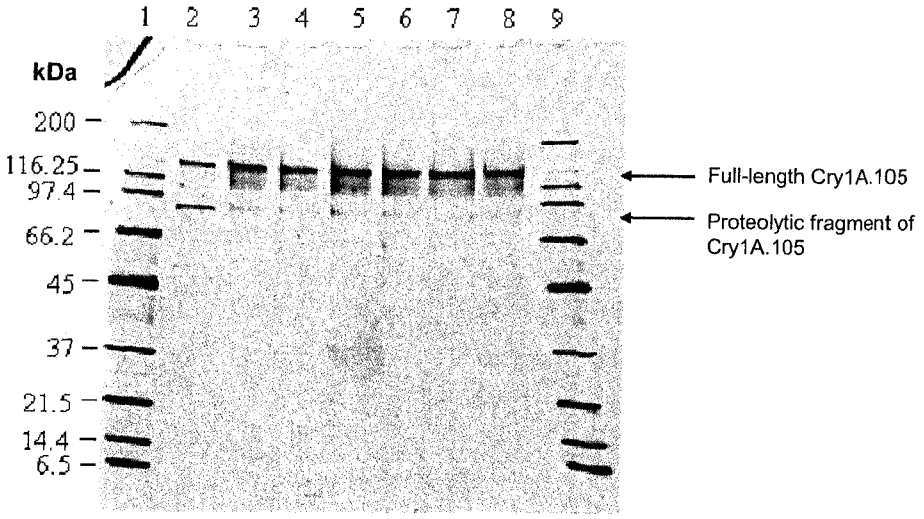

Figure 25. SDS-PAGE of E. coli-and MON 89034-produced Cry1A.105 protein. Aliquots of the MON 89034-produced Cry1A.105 protein and the *E. coli*-produced Cry1A.105 reference standard were separated by a Tris-glycine 4→20% polyacrylamide gradient gel and stained with an Invitrogen SilverXpress silver staining kit. Approximate molecular weights (kDa) are shown on the left side of the gel and correspond to the markers loaded in lanes 1 and 9.

Lane	Sample	Amount (ng)
1	Broad Range molecular weight markers (Bio-Rad)	—
2	*E. coli*-produced Cry1A.105 reference standard	96
3	MON 89034-produced Cry1A.105 protein	48
4	MON 89034-produced Cry1A.105 protein	48
5	MON 89034-produced Cry1A.105 protein	72
6	MON 89034-produced Cry1A.105 protein	72
7	MON 89034-produced Cry1A.105 protein	96
8	MON 89034-produced Cry1A.105 protein	96
9	Broad Range molecular weight markers (Bio-Rad)	—

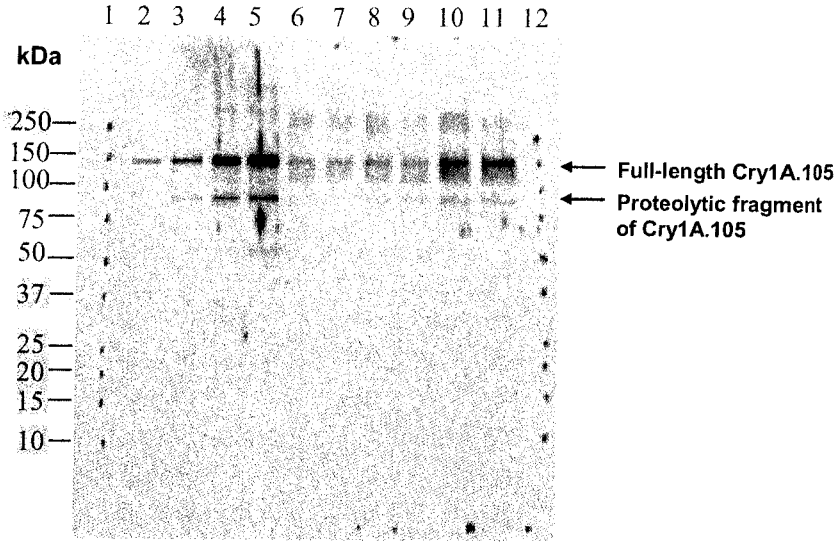

Figure 26. Western blot analysis of MON 89034-produced and *E. coli*-produced Cry1A.105 protein.

Aliquots of the purified, MON 89034-produced and *E. coli*-produced Cry1A.105 protein were separated by SDS-PAGE, and electrotransferred to a PVDF membrane. The membrane was probed with rabbit polyclonal anti-Cry1A.105 antibody and developed using an ECL system (Amersham Pharmacia). Approximate molecular weights (kDa) are shown on the left side of the blot, which correspond to the markers loaded in lanes 1 and 12.

Lane	Sample	Amount Loaded (ng)	Amount Loaded (µl)
1	Precision Plus Dual Colour molecular weight markers	—	—
2	*E. coli*-produced Cry1A.105 standard	1	—
3	*E. coli*-produced Cry1A.105 standard	2	—
4	*E. coli*-produced Cry1A.105 standard	4	—
5	*E. coli*-produced Cry1A.105 standard	6	—
6	MON 89034-produced Cry1A.105 protein	—	7.5
7	MON 89034-produced Cry1A.105 protein	—	7.5
8	MON 89034-produced Cry1A.105 protein	—	10
9	MON 89034-produced Cry1A.105 protein	—	10
10	MON 89034-produced Cry1A.105 protein	—	20
11	MON 89034-produced Cry1A.105 protein	—	20
12	Precision Plus Dual Colour molecular weight markers	—	—

- **Examination of the N-terminus intactness of Cry1A.105 protein**

The N-terminus of the plant-produced proteins could be blocked by chemical modifications which would not allow N-terminal analysis by the typical Edman degradation method (Brown and Roberts, 1976; Arfin and Bradshaw, 1988). Therefore, the intactness of the N-terminus of the MON 89034-produced Cry1A.105 was examined using western blot analysis with an N-terminal peptide-specific antibody. The anti-N-terminal peptide antibody was raised against a synthetic peptide consisting of the first 14 amino acids (MDNNPNINECIPYN) at the N-terminus of the Cry1A.105 protein.

The *E. coli*-produced Cry1A.105 containing the intact N-terminal sequence, and the Cry1A.105 trypsin-resistant core lacking the N-terminus of the full-length protein were used as positive and negative reference standards, respectively. As expected, no immunoreactive bands were observed in the lanes loaded with Cry1A.105 trypsin-resistant core (**Figure 27**, lanes 6 and 7). The band corresponding to the full-length Cry1A.105 protein (approximately 130 kDa) was observed in the lanes loaded with both the MON 89034- and *E. coli*-produced Cry1A.105 protein. As expected, the intensity of the bands increased in a manner dependent of the loading quantities. Additionally, a fragment of lower molecular weight (approximately 85 kDa) was observed in both protein preparations (**Figure 27**, lanes 2-5). This fragment represents a proteolytic product of C-terminal degradation of Cry1A.105 protein. These results are consistent with published literature that the intermediate proteolytic fragments of Cry1 proteins have intact N-terminus, while the N-terminal peptide (approximately 25-30 amino acids) was cleaved for the trypsin-resistant core (Gao et al., 2006).

In conclusion, the intactness of the N-terminus of the MON 89034- and the *E.coli* produced Cry1A.105 protein was confirmed.

- **Tryptic peptide mapping by MALDI-TOF MS**

The MON 89034-produced, full-length Cry1A.105 protein was further identified by tryptic peptide mapping analysis using MALDI-TOF MS. The protein sample was heat-denatured, chemically reduced, alkylated and digested with trypsin, and the masses of the tryptic peptides were measured.

There were 52 protein peptide masses identified that matched the expected tryptic peptides generated *in silico* based on the predicted trypsin cleavage sites in the sequence. The identified masses were used to assemble a coverage map that displays those matched peptide sequences for the protein (**Figure 28**). Overall, the confirmed sequence accounts for 43.8% (516 out of 1,177 amino acids) of the full-length sequence of Cry1A.105 protein. In general, a protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). In the current case, the detected peptide coverage was 43.8% with 52 matched peptides, therefore, the protein identity of the MON 89034-produced Cry1A.105 was confirmed.

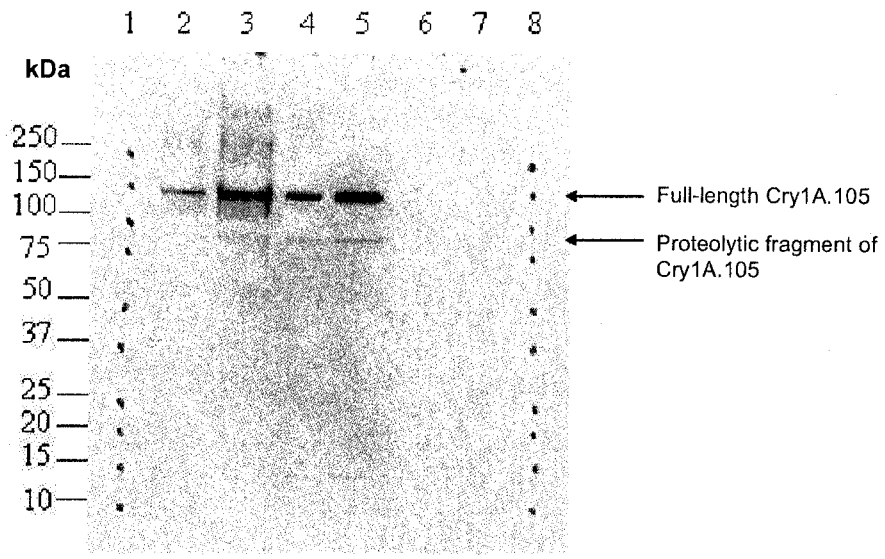

Figure 27. Examination of the intactness of N-terminus by immunoblot analysis using anti-N-terminal peptide antibody.

Aliquots of the MON 89034–produced Cry1A.105 protein, *E. coli*-produced Cry1A.105 reference standard, and Cry1A.105 trypsin-resistant core standard were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was probed with the anti-N-terminal peptide antibody, and immunoreactive bands were visualised using an ECL system (5 min exposure). Approximate molecular weights (kDa) are shown on the left side of the blot and correspond to the markers loaded in lanes 1 and 8.

Lane	Sample	Amount (ng)
1	Precision Plus Dual Colour molecular weight markers	—
2	MON 89034-produced Cry1A.105 protein	20
3	MON 89034-produced Cry1A.105 protein	40
4	*E. coli*-produced Cry1A.105 reference standard	20
5	*E. coli*-produced Cry1A.105 reference standard	40
6	Cry1A.105 trypsin-resistant core standard	20
7	Cry1A.105 trypsin-resistant core standard	40
8	Precision Plus Dual Colour molecular weight markers	—

```
0001  MDNNPNINEC  IPYNCLSNPE  VEVLGGERIE  TGYTPIDISL  SLTQFLLSEF
0051  VPGAGFVLGL  VDIIWGIFGP  SQWDAFLVQI  EQLINQRIEE  FARNQAISRL
0101  EGLSNLYQIY  AESFREWEAD  PTNPALREEM  RIQFNDMNSA  LTTAIPLFAV
0151  QNYQVPLLSV  YVQAANLHLS  VLRDVSVFGQ  RWGFDAATIN  SRYNDLTRLI
0201  GNYTDHAVRW  YNTGLERVWG  PDSRDWIRYN  QFRRELTLTV  LDIVSLFPNY
0251  DSRTYPIRTV  SQLTREIYTN  PVLENFDGSF  RGSAQGIEGS  IRSPHLMDIL
0301  NSITIYTDAH  RGEYYWSGHQ  IMASPVGFSG  PEFTFPLYGT  MGNAAPQQRI
0351  VAQLGQGVYR  TLSSTLYRRP  FNIGINNQQL  SVLDGTEFAY  GTSSNLPSAV
0401  YRKSGTVDSL  DEIPPQNNNV  PPRQGFSHRL  SHVSMFRSGF  SNSSVSIIRA
0451  PMFSWIHRSA  EFNNIIASDS  ITQIPLVKAH  TLQSGTTVVR  GPGFTGGDIL
0501  RRTSGGPFAY  TIVNINGQLP  QRYRARIRYA  STTNLRIYVT  VAGERIFAGQ
0551  FNKTMDTGDP  LTFQSFSYAT  INTAFTFPMS  QSSFTVGADT  FSSGNEVYID
0601  RFELIPVTAT  LEAEYNLERA  QKAVNALFTS  TNQLGLKTNV  TDYHIDQVSN
0651  LVTYLSDEFC  LDEKRELSEK  VKHAKRLSDE  RNLLQDSNFK  DINRQPERGW
0701  GGSTGITIQG  GDDVFKENYV  TLSGTFDECY  PTYLYQKIDE  SKLKAFTRYQ
0751  LRGYIEDSQD  LEIYSIRYNA  KHETVNVPGT  GSLWPLSAQS  PIGKCGEPNR
0801  CAPHLEWNPD  LDCSCRDGEK  CAHHSHHFSL  DIDVGCTDLN  EDLGVWVIFK
0851  IKTQDGHARL  GNLEFLEEKP  LVGEALARVK  RAEKKWRDKR  EKLEWETNIV
0901  YKEAKESVDA  LFVNSQYDQL  QADTNIAMIH  AADKRVHSIR  EAYLPELSVI
0951  PGVNAAIFEE  LEGRIFTAFS  LYDARNVIKN  GDFNNGLSCW  NVKGHVDVEE
1001  QNNQRSVLVV  PEWEAEVSQE  VRVCPGRGYI  LRVTAYKEGY  GEGCVTIHEI
1051  ENNTDELKFS  NCVEEEIYPN  NTVTCNDYTV  NQEEYGGAYT  SRNRGYNEAP
1101  SVPADYASVY  EEKSYTDGRR  ENPCEFNRGY  RDYTPLPVGY  VTKELEYFPE
1151  TDKVWIEIGE  TEGTFIVDSV  ELLLMEE
```

Figure 28. Sequence coverage in the tryptic peptide mapping analysis of MON 89034-produced full-length Cry1A.105 with MALDI-TOF MS.

Shaded regions correspond to 52 fragments of tryptic peptide masses that were identified from the full-length protein band. Overall, 43.8% of the protein sequence was identified.

- **Lack of glycosylation for Cry1A.105 protein**

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple oligosaccharides to monosaccharides. In contrast, prokaryotic organisms such as non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical synthetic capacity required for protein glycosylation. Therefore, determining whether the MON 89034-produced Cry1A.105 protein is equivalent to the *E. coli*-produced Cry1A.105 protein requires an investigation of its glycosylation status.

To assess whether potential post-translational glycosylation of the MON 89034-produced Cry1A.105 protein occurred, the purified protein sample was subjected to glycosylation analysis. The *E.* coli-produced Cry1A.105 reference standard represented a negative control. The positive controls were the transferrin and horse-radish peroxidase (HRP) proteins which are known to have multiple covalently-linked carbohydrate modifications. The transferrin protein and HRP, as well as the purified Cry1A.105 protein isolated from MON 89034 and *E. coli* were separated on SDS-PAGE, and glycosylation analysis was performed to detect oxidised carbohydrate moieties on the proteins. The results of this analysis are shown in **Figure 29**. The positive controls were detected at the expected molecular weights in a concentration-dependent manner (**Figure 29**, lanes 2-5). No detectable signal was observed for the MON 89034-produced and *E. coli*-produced Cry1A.105 protein (**Figure 29**, lanes 6-9).

These results indicate that the MON 89034-produced protein is not glycosylated and, thus is equivalent to the *E. coli*-produced Cry1A.105 reference standard with respect to the lack of glycosylation.

- **Cry1A.105 functional activity**

The biological activities of *E. coli*-produced and MON 89034-produced Cry1A.105 protein were estimated by determining EC_{50} values in a corn earworm (CEW) diet-incorporation bioassay. The EC_{50} value is defined as the level of Cry1A.105 protein in the diet that results in 50% inhibition of larval growth.

The EC_{50} values for each replicate bioassay are summarised in **Table 6**, and the dose response relationships for MON 89034- and *E. coli*-produced Cry1A.105 are illustrated in **Figure 30**. The ranges of the estimated EC_{50} values overlapped for the protein from the two host sources. The EC_{50} values for the MON 89034-produced protein ranged from 0.0055 to 0.0089 µg Cry1A.105/ml diet and the EC_{50} values for the *E. coli*-produced protein ranged from 0.0053 to 0.0170 µg Cry1A.105/ml diet. **Figure 30** shows an equivalent slope for the dose-response relationship for the *E. coli*- and MON 89034-produced Cry1A.105 protein in the CEW bioassay. These results clearly demonstrate that the Cry1A.105 protein derived from MON 89034 and *E. coli* have equivalent functional activities.

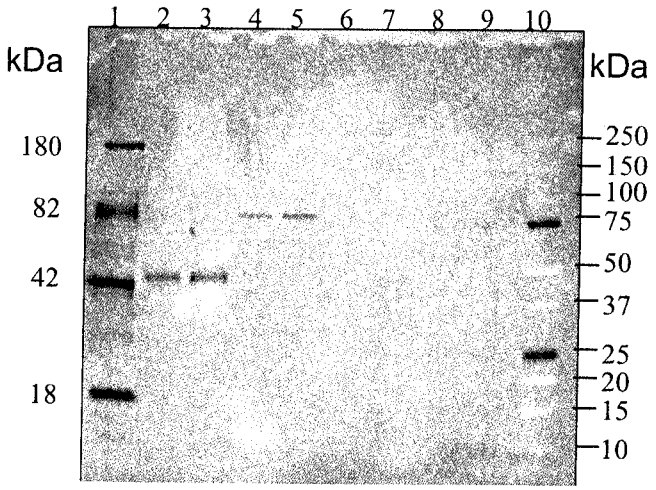

Figure 29. Glycosylation analysis of the MON 89034-produced Cry1A.105 protein.

Aliquots of the MON 89034-produced Cry1A.105 protein, *E. coli*-produced Cry1A.105 reference standard (negative control), horseradish peroxidase (positive control) and transferrin (positive control) were separated by SDS-PAGE (4→20% gradient) and electrotransferred to a PVDF membrane. Where present, periodate-oxidised protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain and emitted a fluorescent signal at 488 nm. The signal was captured using a Bio-Rad Molecular Imager FX. Approximate molecular weights (kDa) correspond to the CandyCane glycoprotein markers loaded in lane 1 and the Precision Dual Colour markers in lane 10.

Lane	Sample	Amount (ng)
1	CandyCane glycoprotein molecular weight standards	—
2	Horseradish Peroxidase (positive control)	48
3	Horseradish Peroxidase (positive control)	96
4	Transferrin (positive control)	48
5	Transferrin (positive control)	96
6	MON 89034-produced Cry1A.105	48
7	MON 89034-produced Cry1A.105	96
8	*E. coli*-produced Cry1A.105 (negative control)	48
9	*E. coli*-produced Cry1A.105 (negative control)	96
10	Precision Plus Dual Colour molecular weight markers	—

Table 6. EC_{50} values of *E. coli*-produced and MON 89034-produced Cry1A.105 protein in a corn earworm diet-incorporation bioassay.

		EC_{50} (µg Cry1A.105/ml diet)[1]	
		***E. coli*-produced**	**MON 89034-produced**
Replicate[2]	1	0.0150 ± 0.0025	0.0055 ± 0.0014
	2	0.0053 ± 0.0022	0.0089 ± 0.0018
	3	0.0170 ± 0.0021	0.0077 ± 0.0012
Overall		0.0120 ± 0.0062	0.0074 ± 0.0017

[1] EC_{50} (mean ± standard error) represents the concentration needed to inhibit the growth of the target insect by 50%.

[2] Each bioassay replicate consisted of a series of five protein levels yielding a dose series ranging from 0.00048 – 0.039 µg Cry1A.105 protein/ml diet with a 3-fold separation factor between dose levels. Insect larvae were placed on the diets with 16 insects per treatment. The combined weight of the surviving insects at each dose level was recorded at the end of the 6-7 day incubation period. EC_{50} was calculated with SAS software.

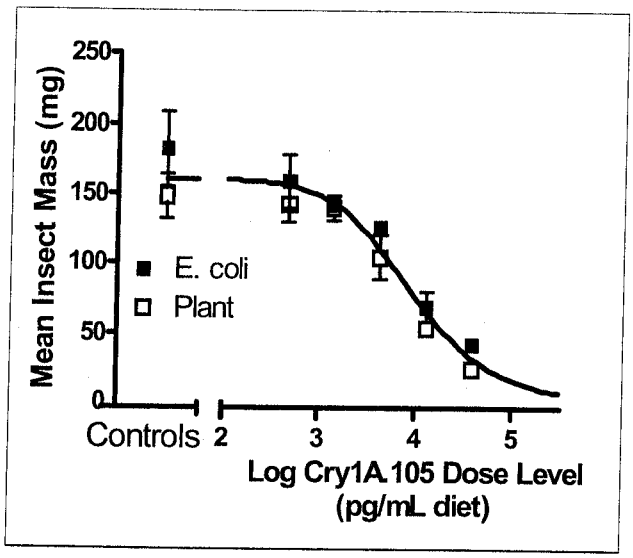

Figure 30. Functional equivalence of the MON 89034-produced and *E. coli*-produced Cry1A.105 protein against corn earworm (CEW).

Bioassay results from three replicates have been combined for illustration purpose only and are shown on a log concentration scale. Each data point represents the mean of the three bioassays along with the standard error of the mean. Equivalent slope was seen for dose-response relationships for the MON 89034-produced and *E. coli*-produced Cry1A.105 protein in the CEW bioassay. The dose response curve was modeled with SAS software. The data demonstrate that the test insect body weight decreases with the increase of the Cry1A.105 dose level, indicating the greater growth inhibitory effect on the test insects at higher doses.

iii) Identity and function of the Cry2Ab2 protein

The Cry2Ab2 is a protein from *Bacillus thuringiensis* subsp. *kurstaki* with a demonstrated history of safe use. Cry2Ab2 protein has 88% amino acid sequence identity to Cry2Aa protein which is present in commercial microbial pest control products such as DiPel® and Crymax®.

Like the Cry2Ab2 protein produced in the biotechnology-derived cotton Bollgard II, the Cry2Ab2 protein produced in maize MON 89034 is a variant of the wild-type Cry2Ab2 protein from *Bt*. Accumulation of the Cry2Ab2 protein in MON 89034 is targeted to the chloroplast using a chloroplast transit peptide (CTP). CTPs facilitate the intracellular transport of proteins from the cytoplasm to the plastids (Bruce, 2000). CTPs are typically cleaved from the mature protein upon translocation into the chloroplast, and then rapidly degraded. To allow targeting of the Cry2Ab2 protein to the plastids in MON 89034, the DNA sequence encoding the CTP region of maize ribulose 1,5-biosphosphate carboxylase small subunit is fused to the *cry2Ab2* coding sequence.

The Cry2Ab2 protein expressed in cotton Bollgard II is also targeted to a plastid through a CTP. The attempts to determine the N-terminal sequence of the Cry2Ab2 protein in cotton Bollgard II (after the CTP was cleaved) indicate that the N-terminus was blocked, and therefore the exact excision site of the CTP is not known. Attempts to determine the N-terminal sequence of the full-length MON 89034-produced Cry2Ab2 also indicated that the protein was blocked at the N-terminus, and as a consequence, the cleavage site in the CTP that is processed in the chloroplast could not be determined. The CTP used in MON 89034 has a potential cleavage site (methionine), located three amino acids upstream from the start of the Cry2Ab2 protein sequence (**Figure 31**). As such, the three additional amino acids from the CTP were included at the N-terminus for the *E. coli*-produced Cry2Ab2 protein used in the safety assessment studies for MON 89034 (**Figure 31**). This resulted in the production of a Cry2Ab2 protein of 637 amino acids (634 from Cry2Ab2 and 3 from the CTP) with a theoretical molecular weight of 71 kDa.

Cry2Ab2 protein is active against maize and cotton pests. The spectrum of activity includes corn borers from the genera *Ostrinia* and *Diatraea* (such as European corn borer, Asian corn borer, sugarcane borer, southwestern corn borer), armyworms (*Spodoptera* spp. including fall armyworm), earworms (*Helicoverpa* spp. including corn earworm), cutworms (e.g., *Agrotis ipsilon*, black cutworm), tobacco budworm (*Heliothis virescens*) and pink bollworm (*Pectinophora gossypiella*).

® DiPel and Crymax are registered trademarks of Abbott and Ecogen, Inc., respectively.

MON 89034	**M-Q-A**[1]-M-**D**[2]- N-S-V-L-N
Recombinant *E. coli*	**M-Q-A**[1]-M-**D**[2]- N-S-V-L-N
B. thuringiensis	-M- - N-S-V-L-N

[1] **M-Q-A** – predicted amino acids from chloroplast transit peptides (CTP)
[2] **D** – an additional amino acid included for the ease of cloning

Figure 31. Comparison of the putative N-terminal sequences of the Cry2Ab2 proteins produced in MON 89034, the recombinant *E. coli*, and *Bacillus thuringiensis*.

iv) Characterisation of the Cry2Ab2 protein

The Cry2Ab2 protein was purified from the grain of MON 89034 maize as well as from an *E. coli* culture. A panel of analytical tests was used to identify, characterise and compare the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein including: (1) western blot analysis; (2) SDS-PAGE; (3) MALDI-TOF MS; (4) N-terminal sequence analysis with Edman degradation chemistry; (5) glycosylation analysis; and (6) insect activity bioassay.

- **Cry2Ab2 protein molecular weight equivalence**

The equivalence in molecular weight of the purified MON 89034- and the *E. coli*-produced Cry2Ab2 protein was demonstrated using SDS-PAGE stained with Brilliant Blue G-Colloidal stain. The MON 89034-produced, full-length Cry2Ab2 protein migrated to a position comparable to that of the *E. coli*-produced protein standard which was analysed concurrently (**Figure 32**). Based on the comparable electrophoretic mobility, the MON 89034- and *E. coli*-produced, full-length Cry2Ab2 protein were determined to have equivalent molecular weight. A band observed below the full-length band is a proteolytic fragment.

- **Cry2Ab2 protein immunoreactivity**

Western blot analysis using a polyclonal anti-Cry2Ab2 antibody was conducted to confirm the identity and determine the relative immunoreactivity of the MON 89034-produced Cry2Ab2 protein and the *E. coli*-produced Cry2Ab2 reference standard. Results indicated that the anti-Cry2Ab2 antibody recognised the MON 89034-produced Cry2Ab2 protein which migrated comparably to the *E. coli*-produced reference standard protein (**Figure 33**, band-1). The immunoreactive signal increased with increasing levels of the Cry2Ab2 protein. Besides the expected band, an immunoreactive band with lower molecular weight (**Figure 33**, band-2) was observed in the partially purified MON 89034-produced Cry2Ab2 sample, which represents a proteolytic fragment of the Cry2Ab2 protein.

The above western blot result confirmed the identity of Cry2Ab2 protein produced by MON 89034, and demonstrated that MON 89034- and *E. coli*-produced Cry2Ab2 had equivalent immunoreactivity with anti-Cry2Ab2 antibody.

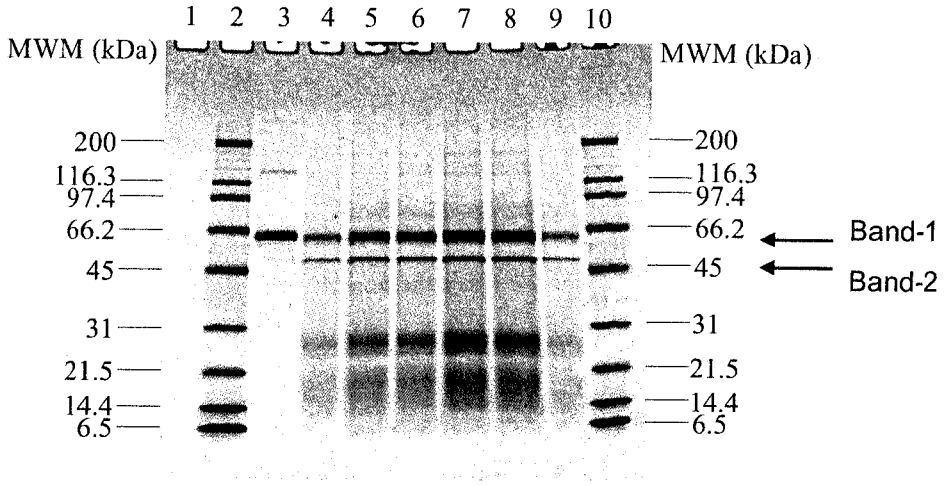

Figure 32. SDS-PAGE of the MON 89034- and E. coli-produced Cry2Ab2 protein.

Samples of the partially purified MON 89034-produced Cry2Ab2 protein, and the *E. coli*-produced Cry2Ab2 reference standard were separated by a Tris-glycine 4→20% SDS polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Amounts loaded correspond to total protein loaded per lane. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.

Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MWM (molecular weight markers, Bio-Rad, broad range)	4.5
3	*E. coli*-produced Cry2Ab2 reference standard	1
4	MON 89034-produced Cry2Ab2 protein	1
5	MON 89034-produced Cry2Ab2 protein	2
6	MON 89034-produced Cry2Ab2 protein	2
7	MON 89034-produced Cry2Ab2 protein	3
8	MON 89034-produced Cry2Ab2 protein	3
9	MON 89034-produced Cry2Ab2 protein	1
10	MWM (molecular weight markers, Bio-Rad, broad range)	4.5

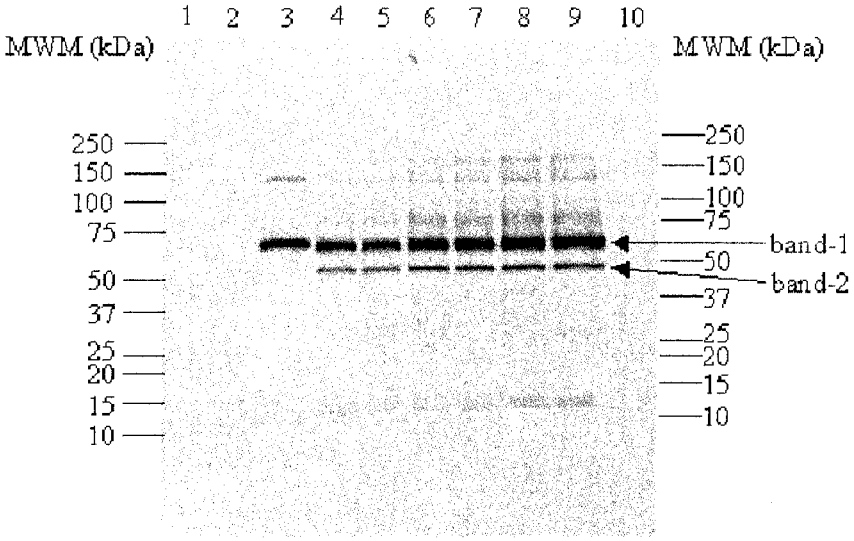

Figure 33. Western blot analysis of MON 89034-produced and *E. coli*-produced Cry2Ab2 protein.

Samples of the partially purified MON 89034-produced and *E. coli*-produced Cry2Ab2 protein were separated by SDS-PAGE (4→20% gradient), electrotransferred to a PVDF membrane. The membrane was then detected using goat anti-Cry2Ab2 antibody and developed using an ECL system. Amounts loaded correspond to subject protein after normalisation with purity. The approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.

Lane	Sample	Amount of Cry2Ab2 (ng)
1	Empty Lane	N/A
2	Precision Plus Dual Colour molecular weight markers (MWM)	N/A
3	*E. coli*-produced Cry2Ab2 protein	20
4	MON 89034-produced Cry2Ab2 protein	20
5	MON 89034-produced Cry2Ab2 protein	20
6	MON 89034-produced Cry2Ab2 protein	30
7	MON 89034-produced Cry2Ab2 protein	30
8	MON 89034-produced Cry2Ab2 protein	40
9	MON 89034-produced Cry2Ab2 protein	40
10	Precision Plus Dual Colour molecular weight markers (MWM)	N/A

- **Analysis of the N-terminal sequence**

N-terminal sequencing analysis demonstrated that the MON 89034-produced, full-length Cry2Ab2 (band-1) was blocked at the N-terminus with no definitive sequence obtained in the sequencing analysis using Edman degradation chemistry. A minor portion of the protein co-migrating with the full-length protein was proteolytically degraded and the sequence was determined to start from amino acid residue No. 24. N-terminal sequence analysis of the lower molecular weight proteolytic fragment (band-2) revealed that this fragment starts from amino acid residue No. 145. With *E. coli*-produced Cry2Ab2 (band-1) the N-terminus sequence was determined as MQAMDN, as expected. This result further confirms that band-1 is the full-length Cry2Ab2 protein.

In summary, the N-terminal sequencing results indicate that MON 89034-produced, full-length Cry2Ab2 protein is blocked at its N-terminus, but the N-terminal sequencing result of the lower molecular weight proteolytic fragment (band-2) confirms the Cry2Ab2 identity. In addition, the N-terminus of *E. coli*-produced, full-length Cry2Ab2 was confirmed.

- **Tryptic peptide mapping by MALDI-TOF MS**

The MON 89034-produced, full-length Cry2Ab2 (band-1, in **Figure 33**) and the proteolytic fragment (band-2, in **Figure 33**) were characterised by tryptic peptide mapping analysis with MALDI-TOF MS to further confirm their identity. For band-1, a total of 32 observed peptide masses matched the theoretical tryptic peptide masses of Cry2Ab2 protein. These identified peptides were used to assemble a coverage map in the Cry2Ab2 protein sequence (**Figure 34**). The overall peptide sequence coverage was 44.4% out of the 637 amino acid residues of the full-length Cry2Ab2 protein. For band-2, a total of 24 observed peptide masses matched the expected tryptic peptide masses of Cry2Ab2 protein, which yielded a coverage map equal to 47.7% out of the 493 amino acid residues of this proteolytic fragment (**Figure 35**).

In general, a protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). In the current case, the detected peptide coverage was 44.4% with 32 matched peptides for the full-length Cry2Ab2 protein, and 47.7% with 24 matched peptides for the proteolytic fragment of Cry2Ab2, therefore, the identity of MON 89034-produced Cry2Ab2 protein is confirmed.

```
  1 MQAMDNSVLN SGRTTICDAY NVAAHDPFSF QHKSLDTVQK EWTEWKKNNH SLYLDPIVGT
 61 VASFLLKKVG SLVGKRILSE LRNLIFPSGS TNLMQDILRE TEKFLNQRLN TDTLARVNAE
121 LTGLQANVEE FNRQVDNFLN PNRNAVPLSI TSSVNTMQQL FLNRLPQFQM QGYQLLLLPL
181 FAQAANLHLS FIRDVILNAD EWGISAATLR TYRDYLKNYT RDYSNYCINT YQSAFKGLNT
241 RLHDMLEFRT YMFLNVFEYV SIWSLFKYQS LLVSSGANLY ASGSGPQQTQ SFTSQDWPFL
301 YSLFQVNSNY VLNGFSGARL SNTFPNIVGL PGSTTTHALL AARVNYSGGI SSGDIGASPF
361 NQNFNCSTFL PPLLTPFVRS WLDSGSDREG VATVTNWQTE SFETTLGLRS GAFTARGNSN
421 YFPDYFIRNI SGVPLVVRNE DLRRPLHYNE IRNIASPSGT PGGARAYMVS VHNRKNNIHA
481 VHENGSMIHL APNDYTGFTI SPIHATQVNN QTRTFISEKF GNQGDSLRFE QNNTTARYTL
541 RGNGNSYNLY LRVSSIGNST IRVTINGRVY TATNVNTTTN NDGVNDNGAR FSDINIGNVV
601 ASSNSDVPLD INVTLNSGTQ FDLMNIMLVP TNISPLY
```

Figure 34. Peptide mass coverage map of the MON 89034-produced full-length Cry2Ab2 protein in MALDI-TOF MS analysis.

Shaded regions correspond to 32 fragments of tryptic peptide masses that were identified from the band-1 (**Figure 33**) of the isolated Cry2Ab2 protein from MON 89034. MQA sequence (underlined) originates from the chloroplast transit peptide (CTP).

```
145 AVPLSITSSV NTMQQLFLNR LPQFQMQGYQ LLLLPLFAQA ANLHLSFIRD VILNADEWGI
205 SAATLRTYRD YLKNYTRDYS NYCINTYQSA FKGLNTRLHD MLEFRTYMFL NVFEYVSIWS
265 LFKYQSLLVS SGANLYASGS GPQQTQSFTS QDWPFLYSLF QVNSNYVLNG FSGARLSNTF
325 PNIVGLPGST TTHALLAARV NYSGGISSGD IGASPFNQNF NCSTFLPPLL TPFVRSWLDS
385 GSDREGVATV TNWQTESFET TLGLRSGAFT ARGNSNYFPD YFIRNISGVP LVVRNEDLRR
445 PLHYNEIRNI ASPSGTPGGA RAYMVSVHNR KNNIHAVHEN GSMIHLAPND YTGFTISPIH
505 ATQVNNQTRT FISEKFGNQG DSLRFEQNNT TARYTLRGNG NSYNLYLRVS SIGNSTIRVT
565 INGRVYTATN VNTTTNNDGV NDNGARFSDI NIGNVVASSN SDVPLDINVT LNSGTQFDLM
625 NIMLVPTNIS PLY
```

Figure 35. Peptide mass coverage map of the MON 89034-derived proteolytic fragment of Cry2Ab2 protein in MALDI-TOF MS analysis.

Shaded regions correspond to 24 tryptic peptide masses that were identified from the band-2 proteolytic fragment of Cry2Ab2 (**Figure 33**) from MON 89034. The amino acid residue number was assigned based on the respective position in the full-length sequence of Cry2Ab2 protein. N-terminal sequencing result showed that band-2 started from amino acid residue No. 145.

- **Lack of glycosylation of MON 89034-produced Cry2Ab2**

Some eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple oligosaccharides to monosaccharides. In contrast, prokaryotic organisms such as non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical "machinery" required for protein glycosylation. Therefore, determining whether the MON 89034-produced Cry2Ab2 protein is equivalent to the *E. coli*-produced Cry2Ab2 protein requires an investigation of its glycosylation status.

To determine whether post-translational glycosylation of the MON 89034-produced Cry2Ab2 protein occurred, the isolated MON 89034-produced Cry2Ab2 protein was analysed for the presence of covalently bound carbohydrate moieties. The *E. coli*-produced Cry2Ab2 reference standard (negative control) and transferrin (positive control) were analysed concurrently with the MON 89034-produced Cry2Ab2 protein.

The result showed that positive glycoprotein transferrin was detected, as expected, in a concentration-dependent manner at loadings of 0.5 and 1.0 µg/lane (**Figure 36,** lanes 3-4). No detectable signal was observed for the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein at the positions of the expected molecular weights between 50 to 75 kDa (**Figure 36,** lanes 5-8).

Since no positive signal was seen at the expected band position for Cry2Ab2 protein, it was evident that the MON 89034-produced Cry2Ab2 protein is not glycosylated and is equivalent to the *E. coli*-produced Cry2Ab2 protein regarding to the lack of glycosylation.

- **Functional activity of the Cry2Ab2 protein**

The functional activity of the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein was compared by determining EC_{50} values in a corn earworm diet-incorporation bioassay. The EC_{50} value is defined as the level of Cry2Ab2 protein in the diet that results in 50% inhibition to larval growth.

The EC_{50} values for each replicate bioassay are summarised in **Table 7** and the dose response relationships for the Cry2Ab2 protein from the two sources are illustrated in **Figure 37**. The mean EC_{50} values for the *E. coli*- and the MON 89034-produced proteins were similar and were estimated to be 0.16 µg Cry2Ab2/ml diet, with standard errors of 0.04 and 0.01 µg Cry2Ab2/ml diet, respectively. **Figure 37** shows an equivalent slope for the dose-response relationship for the *E. coli*- and MON 89034-produced Cry2Ab2 protein in the CEW bioassay. These results clearly showed that MON 89034- and *E. coli*-produced Cry2Ab2 protein have equivalent functional activities.

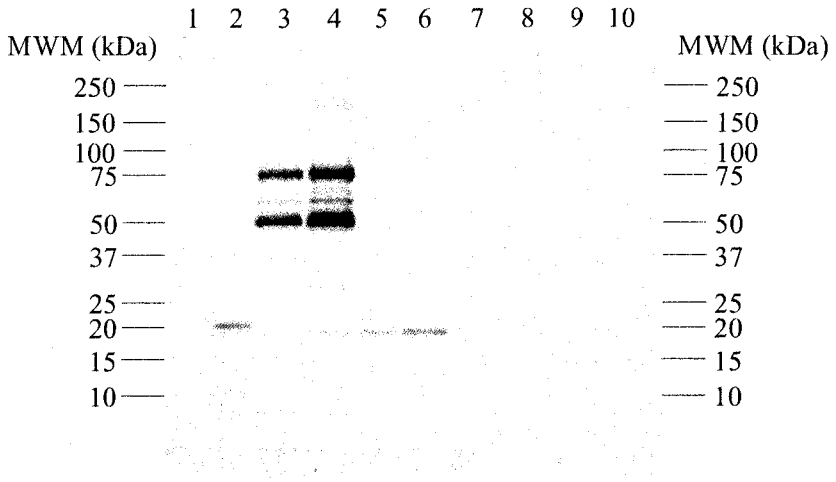

Figure 36. Glycosylation analysis of the MON-89034-produced Cry2Ab2 protein.

Samples of the MON 89034-produced Cry2Ab2 protein, *E. coli*-produced Cry2Ab2 reference standard (negative control) and transferrin (positive control) were separated by a Tris-glycine 4→20% SDS polyacrylamide gel, and electrotransferred to PVDF membrane. Where present, protein-bound carbohydrate moieties were labelled with biotin, and detected with streptavidin-horseradish peroxidase and enhanced chemiluminescence. Amount refers to total protein loaded per lane, except for the *E. coli* and the MON 89034-produced proteins whose concentrations were normalised based on Cry2Ab2 purity.

Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MWM (molecular weight markers, Precision Plus Dual Colour)	N/A
3	Transferrin (positive control)[1]	0.5
4	Transferrin (positive control)[1]	1
5	*E. coli*-produced Cry2Ab2 protein (negative control)	0.5
6	*E. coli*-produced Cry2Ab2 protein (negative control)	1
7	MON 89034-produced Cry2Ab2 protein	0.5
8	MON 89034-produced Cry2Ab2 protein	1
9	Empty Lane	N/A
10	Empty Lane	N/A

[1] Part of the transferrin appeared to be degraded. But this did not affect the utility of this protein as a positive control for glycoproteins in this test.

Table 7. EC_{50} values of *E. coli*- and MON 89034-produced Cry2Ab2 proteins in a corn earworm diet-incorporation bioassay.

		EC_{50} (µg Cry2Ab2/ml diet)[1]	
		***E. coli*-produced**	**MON 89034-produced**
Replicate[2]	1	0.13 ± 0.03	0.17 ± 0.03
	2	0.16 ± 0.02	0.16 ± 0.03
	3	0.20 ± 0.02	0.16 ± 0.02
Overall		0.16 ± 0.04	0.16 ± 0.01

[1] EC_{50} (mean ± standard error) represents the concentration needed to inhibit the growth of the target insect by 50%.

[2] Each bioassay replicate for the *E. coli*-produced and MON 89034-produced Cry2Ab2 proteins consisted of a series of seven dilutions yielding a dose series with a 2-fold separation factor ranging from 0.016 – 1.0 µg Cry2Ab2 protein/ml diet. Insect larvae were placed on the diets with 16 insects per treatment. The combined weight of the surviving insects at each dose level was recorded at the end of the 7 day incubation period. EC_{50} was calculated with SAS software.

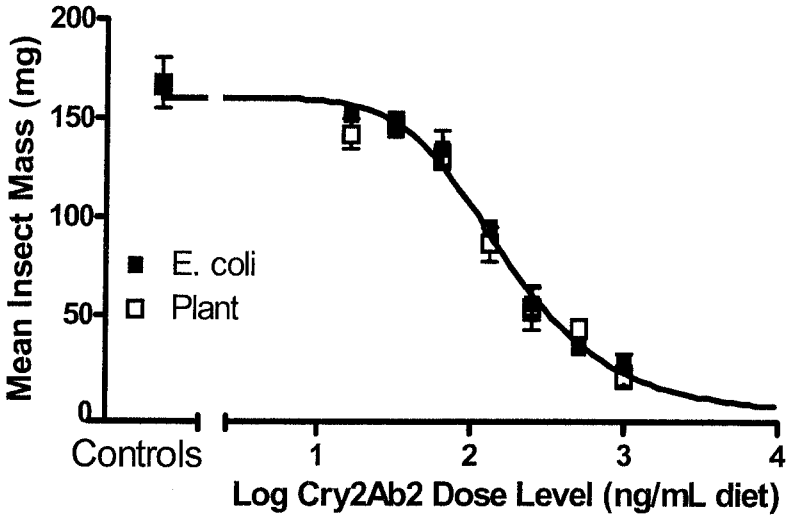

Figure 37. Functional equivalence of the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein against corn earworm (CEW).

Bioassay results from three replicates have been combined for illustration purpose only and are shown on a log concentration scale. Each data point represents the mean of the three bioassays along with the standard error of the mean. Equivalent slope was seen for dose-response relationships for the *E. coli*- and MON 89034-produced Cry2Ab2 protein in the CEW bioassay. The dose response curve was modelled with SAS software. The data demonstrate that the test insect body weight decreases with the increase of the Cry2Ab2 dose level, indicating the greater growth inhibitory effect on the test insects at higher doses.

b) Levels of the Cry1A.105 and Cry2Ab2 Proteins in MON 89034

The levels of the Cry1A.105 and Cry2Ab2 proteins in various tissues of MON 89034 that are relevant to the risk assessment were assessed by validated enzyme-linked immunosorbent assay (ELISA). Tissue samples for analysis were collected from five field trials conducted in the U.S. during 2005. The trial locations were in the states of Iowa, Illinois, Ohio, and Nebraska, which represent the major maize-growing region of the U.S. and provide a range of environmental conditions that would be encountered in the commercial production of maize. At each site, three replicated plots of MON 89034 and a conventional control hybrid were planted using a randomised complete block field design.

Overseason leaf (OSL), overseason whole plant (OSWP), overseason root (OSR), pollen, silk, forage, forage root, grain, stover, and senescent root tissues were collected from each replicated plot at all field sites. The over season leaf and whole plant samples were collected four times at different growth stages: (1) V2 – V4 stage (21 – 29 days after planting), (2) V6 – V8 stage (28 – 43 days after planting), (3) V10 – V12 stage (41 – 53 days after planting), and (4) pre-VT stage (56 – 68 days after planting). The overseason root samples were collected at: (1) V2 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, (4) pre-VT stage, (5) early dent stage (100 – 120 days after planting), and (6) after harvest (130 – 160 days after planting). Pollen and silks were collected approximately 60 – 74 days after planting, forage was collected at approximately 100 – 120 days after planting and grain was harvested at maturity. Stovers were collected following harvest at approximately 130 – 160 days after planting.

The results obtained from ELISA analysis are summarised in **Table 8** for the various tissue types and in **Tables 9** and **10** for the tissues collected throughout the growing season. Cry1A.105 and Cry2Ab2 proteins levels are provided in µg/g fresh weight tissue (fwt) and µg/g dry weight tissue (dwt).

The mean Cry1A.105 levels across sites were: 520 µg/g dwt in young leaf (OSL-1), 12 µg/g dwt in pollen, 26 µg/g dwt in silk, 42 µg/g dwt in forage, 12 µg/g dwt in forage root, 5.9 µg/g dwt in grain, 50 µg/g dwt in stover, and, 11 µg/g dwt in senescent root. In overseason tissues, Cry1A.105 levels across all sites ranged from 27-850 µg/g dwt in leaf, 20-570 µg/g dwt in whole plant, and 6.2-110 µg/g dwt in root. In general, Cry1A.105 levels declined over the growing season.

The mean Cry2Ab2 levels across sites were: 180 µg/g dwt in young leaf, 0.64 µg/g dwt in pollen, 71 µg/g dwt in silk, 38 µg/g dwt in forage, 21 µg/g dwt in forage root, 1.3 µg/g dwt in grain, 62 µg/g dwt in stover, and, 26 µg/g dwt in senescent root. In overseason tissues, Cry2Ab2 levels across all sites ranged from 48-270 µg/g dwt in leaf, 5-230 µg/g dwt in whole plant, and 13-100 µg/g dwt in root. In general, Cry2Ab2 levels declined over the growing season.

Table 8. Cry1A.105 and Cry2Ab2 Protein Levels in MON 89034 Tissues

Tissue Type	Growth Stage	Cry1A.105[2] Mean (SD) [Range], n=15		Cry2Ab2[2] Mean (SD) [Range], n=15	
		µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt
Young leaf	V2-V4	85 (21) 56 – 130	520 (130) 380 - 850	29 (6.8) 19 - 43	180 (59) 94 - 270
Pollen	R1	6.4 (1.5) 3.8 – 8.8	12 (1.7) 8.5 - 16	0.34 (0.084) 0.21 – 0.47	0.64 (0.091) 0.49 - 0.79
Silk	R1	3.0 (0.57) 2.0 – 3.8	26 (3.9) 20 - 31	8.2 (3.6) 3.3 - 16	71 (35) 33 - 160
Forage	R4-R6 (early dent)	14 (3.6) 8.3 – 24	42 (9.4) 20 - 56	12 (4.0) 6.5 - 18	38 (14) 15 - 55
Forage root	R4-R6 (early dent)	2.2 (0.35) 1.3 – 2.7	12 (3.1) 6.2 - 16	4.1 (1.4) 2.2 – 6.5	21 (5.9) 14 - 33
Grain	R6	5.1 (0.67) 4.1 – 6.0	5.9 (0.77) 4.7 - 7.0	1.1 (0.31) 0.67 – 1.8	1.3 (0.36) 0.77 – 2.1
Stover	R6 (after harvest)	17 (4.4) 9.5 – 26	50 (17) 26 - 85	22 (3.6) 17 - 29	62 (15) 46 - 97
Senescent root	R6 (after harvest)	2.2 (0.36) 1.7 – 3.1	11 (1.4) 9.4 - 15	5.3 (2.0) 2.4 – 9.1	26 (8.8) 13 - 43

[1] Young leaf = overseason leaf 1 (OSL-1); n = number of samples; SD = standard deviation; fwt = fresh weight tissue; dwt = dry weight tissue.

[2] Limits of detection (LOD) and limits of quantitation (LOQ) in the various tissues were as follows:

	Cry1A.105 (µg/g fwt)		Cry2Ab2 (µg/g fwt)	
Tissue	LOD	LOQ	LOD	LOQ
Forage	0.372	0.44	0.191	0.44
Leaf	0.568	0.66	0.081	0.44
Pollen	0.412	1.1	0.055	0.11
Root	0.254	0.33	0.056	0.22
Silk	0.275	0.44	0.040	0.22
Grain	0.262	1.1	0.123	0.22

Whole plant and stover were analysed in the same manner as forage and senescent root was analysed in the same manner as root.

Table 9. Cry1A.105 Proteins Levels in Overseason Tissues of MON 89034

Over-Season Tissue (n = 15)	Units[2]	Plant growth stages[1]											
		V2-V4 (21-29 DAP)		V6-V8 (28-43 DAP)		V10-V12 (41-53 DAP)		pre-VT (56-68 DAP)		R4-R6 (100-120 DAP)		R6 (130-160 DAP)	
		µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt
Leaf	Mean (SD)	520 (130)	85 (21)	140 (36)	28 (8.7)	72 (14)	16 (4.3)	120 (77)	30 (20)	N/A	N/A	N/A	N/A
	Range	380-850	56-130	80-200	12-45	47-89	9.4-24	27-240	6.3-59	N/A	N/A	N/A	N/A
Whole plant	Mean (SD)	380 (90)	40 (5.7)	260 (52)	24 (3.7)	100 (26)	11 (2.4)	120 (29)	17 (3.7)	42 (9.4)	14 (3.6)	50 (17)	17 (4.4)
	Range	230-570	30-52	170-350	16-31	58-160	7.0-15	58-170	9.3-22	20-56	8.3-24	26-85	9.5-26
Root	Mean (SD)	79 (17)	8.9 (1.3)	48 (11)	5.8 (1.6)	45 (10)	6.4 (1.8)	42 (10)	6.7 (0.63)	12 (3.1)	2.2 (0.35)	11 (14)	2.2 (0.36)
	Range	52-110	7.3-12	30-63	3.0-8.5	26-64	4.4-10	30-63	5.6-8.1	6.2-16	1.3-2.7	9.4-15	1.7-3.1

[1] Growth stages V2-V4, V6-V8, V10-V12 and pre-VT (tasseling) correspond to overseason tissue samples –1, -2, -3 and –4, respectively (e.g., OSWP-1, etc.). The whole plant and root samples collected at the R4-R6 (early dent) and R6 (after harvest) stages correspond to forage plant/root and stover/senescent root, respectively. N/A - not applicable; VT-vegetative stage at tasseling; DAP - days after planting; dwt - dry weight tissue; fwt - fresh weight tissue.
[2] The LODs and LOQs for Cry1A.105 can be found in **Table 8**. SD = standard deviation.

Table 10. Cry2Ab2 Proteins Levels in Overseason Tissues of MON 89034

Over-Season Tissue (n = 15)	Units[2]	Plant growth stages[1]											
		V2-V4 (21-29 DAP)		V6-V8 (28-43 DAP)		V10-V12 (41-53 DAP)		pre-VT (56-68 DAP)		R4-R6 (100-120 DAP)		R6 (130-160 DAP)	
		µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt
Leaf	Mean (SD)	180 (59)	29 (6.8)	170 (34)	32 (5.3)	130 (34)	29 (5.4)	160 (44)	37 (12)	N/A	N/A	N/A	N/A
	Range	94-270	19-43	110-230	23-44	85-200	23-41	48-210	11-56	N/A	N/A	N/A	N/A
Whole plant	Mean (SD)	130 (51)	13 (4.6)	79 (18)	7.5 (1.8)	40 (9.9)	4.2 (0.94)	39 (16)	5.9 (2.6)	38 (14)	12 (4.0)	62 (15)	22 (3.6)
	Range	52-230	5.2-21	45-110	4.0-9.7	22-61	2.4-5.8	5.0-67	0.7-11	15-55	6.5-18	46-97	17-29
Root	Mean (SD)	56 (17)	6.4 (1.6)	58 (18)	7.6 (4.2)	35 (17)	5.0 (7.7)	26 (5.9)	4.2 (1.2)	21 (5.9)	4.1 (1.4)	26 (8.8)	5.3 (2.0)
	Range	33-100	4.4-10	25-86	2.5-15	15-74	2.2-12	15-45	3.2-7.6	14-33	2.2-6.5	13-43	2.4-9.1

[1] Growth stages V2-V4, V6-V8, V10-V12 and pre-VT (tasseling) correspond to overseason tissue samples –1, -2, -3 and –4, respectively (e.g., OSWP-1, etc.). The whole plant and root samples collected at the R4-R6 (early dent) and R6 (after harvest) stages correspond to forage plant/root and stover/senescent root, respectively. N/A - not applicable; VT-vegetative stage at tasseling; DAP - days after planting; dwt - dry weight tissue; fwt - fresh weight tissue.
[2] The LODs and LOQs for Cry1A.105 can be found in **Table 8**. SD = standard deviation.

c) Similarity of Cry1A.105 and Cry2Ab2 proteins to proteins with a Long History of Safe Consumption

i) Structural similarity of the Cry1A.105 protein with Cry1A class of Bt proteins

The Cry1A.105 protein is a chimeric protein that consists of domains I and II from Cry1Ac or Cry1Ab, domain III from Cry1F, and of the C-terminal domain from Cry1Ac. Cry1Ab and Cry1Ac proteins share 100% amino acid sequence identity in domains I and II. Overall amino acid sequence identity of the Cry1A.105 protein to the Cry1Ac, Cry1Ab, and Cry1F proteins is 93.6%, 90.0%, and 76.7%, respectively. Domain exchange is a well known naturally occurring mechanism for generating diversity and has been described in detail in the literature (De Maagd et al., 2001; Masson et al., 2002).

To better understand the structural relatedness of the Cry1A.105 protein to other Cry proteins, a phylogenetic tree (**Figure 38**) was produced using the Cry1A.105 amino acid sequence and the methods employed to define the Bt protein nomenclature (Crickmore, 2004; Crickmore et al., 1998). The analysis demonstrates that Cry1A.105 belongs to the Cry1A class of Bt proteins and is most closely related (93.6% identical) to the Cry1Ac protein.

Recombinant DNA techniques have been used to generate Bt strains with enhanced insecticidal activity for use as biopesticides (Baum, 1998; Baum et al., 1999). For example, the microbial pesticide Lepinox™ WDG (Ecogen Inc.) that contains a Cry1Ac/Cry1F chimeric protein has been approved and used for control of lepidopteran pests since 1997 (EPA, 1997; Baum, 1998; Baum et al., 1999).

Cry1A proteins are widely used as topical bioinsecticides and in biotechnology-derived crops. FSANZ has completed safety assessments of maize that produces the Cry1Ab (YieldGard®, Bt11) and Cry1F (Herculex® I) proteins, as well as cotton producing the Cry1Ac (Bollgard® and Bollgard II®, Widestrike®) and Cry 1F (Widestrike®) proteins.

The detailed human and animal safety assessments and a history of safe use conducted on Cry1Ab, Cry1Ac, and Cry1F proteins confirm their safety. Due to the chimeric nature of Cry1A.105 which was derived from domains of Cry1Ab, Cry1Ac, and Cry1F, a similar safety profile is expected for Cry1A.105.

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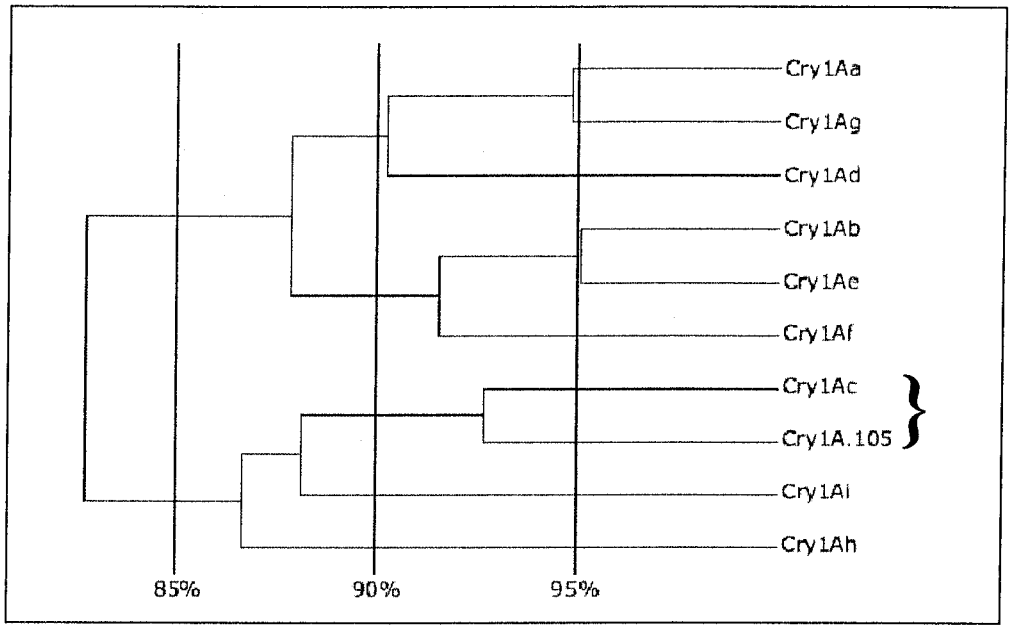


Figure 38. Phylogram establishing that the Cry1A.105 and Cry1Ac proteins are located in the same cluster based on amino acid sequence identity (Crickmore, 2004; Crickmore et al., 1998).

ii) Functional similarity of the Cry1A.105 Protein to the Cry1A class of Bt proteins

Having established the high structural similarity between the Cry1A.105 protein and the Cry1A class of proteins, it is important also to establish the functional similarity. To assess the function of this protein, the biological activity of Cry1A.105 protein was compared to the related proteins Cry1Ab, Cry1Ac, and Cry1F. Amino acid sequence identity for insecticidal proteins predict similarity in biological function, i.e., activity towards a similar spectrum of insects.

It has been established that Cry proteins have a defined spectrum of insecticidal activity within a particular insect order (Crickmore et al., 1998; De Maagd et al., 2001). This high degree of specificity is governed by four levels of selectivity, which collectively lead to intoxication (Federici, 2002). The levels of selectivity include: 1) the route by which the insect is exposed to Cry proteins (i.e., ingestion of plant tissues); 2) protein toxin activation by specific proteolytic enzymes (determined by differences in gut physiology between insects); 3) toxin binding to available specific midgut receptors; and 4) changes in the protein configuration. The reconfigured protein then is able to enter the midgut membrane and form channels. This activity affects larval ability to feed and develop, and eventually leads to death of the susceptible insect.

As a consequence, only insects with specific receptors are affected and no toxicity is observed in species that lack these receptors. For example, the Cry1Ab, Cry1Ac and Cry1F proteins are active against lepidopteran but not coleopteran insects, and the Cry3Bb1 protein is active against coleopteran insects such as corn rootworms but not lepidopteran insects.

Studies were conducted to evaluate the spectrums of the insecticidal activities of the Cry1A.105 protein with a variety of agronomically important insects from three major taxa. Insect species tested included four representative lepidopterans: black cutworm, corn earworm, fall armyworm, and European corn borer; two representative coleopterans: boll weevil (*Anthonomus grandis grandis*) and southern corn rootworm (*Diabrotica undecimpunctata howardi*); and two representative hemipterans: western tarnished plant bug (*Lygus hesperus*) and green peach aphid (*Myzus persicae*). The insects were exposed to high concentrations of Cry1A.105 protein (up to 80 or 100 µg of protein per ml of insect diet in diet-incorporation bioassays). The results showed that Cry1A.105 had activities toward all four representative lepidopteran insects. However, there was no indication of activity by Cry1A.105 against the two coleopteran or two hemipteran representatives. This activity spectrum of Cry1A.105 is similar to the activities of the Cry1Ac, Cry1Ab, and Cry1F proteins described in the literature (De Maagd et al., 2001 and 2003). Similarity in biochemical and functional characteristics between proteins with established safety is a good indication of the general safety of that particular class.

iii) Similarity of the Cry2Ab2 protein with proteins with a history of safe use and consumption

The amino acid sequence of the Cry2Ab2 protein expressed in MON 89034 is 88% identical to the Cry2Aa protein produced by the *Bacillus thuringiensis* subsp. *kurstaki*. The *B. thuringiensis* subsp. *kurstaki* strain controls insect pests by the production of crystalline insecticidal proteins known as delta-endotoxins. These proteins are produced as the bacterium enters the sporulation phase and can account for approximately one-third of the weight of the bacterial cell. These spores are commonly used in agriculture to control insect pests.

In addition to the history of safe use in bacterial sprays, the Cry2Ab2 protein produced in MON 89034 is identical in amino acid sequence to the Cry2Ab2 produced in insect protected cotton Bollgard II. FSANZ approved the sale and use of Bollgard II in Australia and New Zealand in 2002.

d) Evaluation of acute oral toxicity of Cry1A.105 and Cry2Ab2 proteins by mouse gavage

i) Acute oral toxicity study with the Cry1A.105 protein

An oral acute toxicity study was conducted with *E. coli*-produced Cry1A.105 protein. The *E. coli*-produced Cry1A.105 protein was shown to be equivalent to the MON 89034-produced Cry1A.105. Acute administration was considered appropriate to assess the potential toxicity of Cry1A.105 protein because toxic proteins generally act via acute mechanisms (Pariza and Foster, 1983; Sjoblad et al., 1992; Pariza and Johnson, 2001).

Two groups of ten male and ten female young adult CD1 mice received an acute high dose of the Cry1A.105 protein by oral gavage. The target dose of 2072 mg/kg body weight was based on the maximum attainable Cry1A.105 concentration of the dosing solution (estimated at 34.1 mg/mL) and a total dose volume of 66.6 mL/kg body weight. The limited solubility of the Cry1A.105 protein precluded its administration as a single dose. Therefore, dosing was subdivided into two doses of 33.3 mL/kg body weight (66.6 mL/kg total) to achieve the target dose of 2072 mg/kg body weight. On the day of dosing (Day 0) the two individual doses of 33.3 mg/mL body weight were administered about four hours apart. A separate group of ten male and ten female served as protein control group and received bovine serum albumin (BSA) at a dose of 1998 mg/kg body weight. In addition, the vehicle control (buffer) groups of ten males and ten females received the dosing vehicle substance only.

Following dosing, all mice were observed daily, and body weights and food consumption were measured weekly. A gross necropsy examination was performed on all animals at the time of death or the end of the study (day 14).

No test article related mortality or clinical observation was observed. There were no significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and the

Cry1A.105 treated group. No treatment–related gross pathological findings were observed at necropsy.

Under the conditions of this test, no test article related mortality or other toxicity was observed in the Cry1A.105 treated group. Therefore, the acute oral LD_{50} of the Cry1A.105 protein in mice is greater than 2072 mg/kg body weight. This dose was determined to be the no-observed effect level (NOEL) in this study.

ii) Acute oral toxicity study with Cry2Ab2 protein

An acute oral toxicity assessment was conducted to evaluate potential adverse effects on mice as a result from exposure to *E. coli*-produced Cry2Ab2 protein. The Cry2Ab2 protein produced in *E. coli* was shown to be equivalent to the MON 89034-produced Cry2Ab2. The results of this study were consistent with the study conducted for the Cry2Ab2 protein produced in insect-protected cotton Bollgard II (Hileman and Astwood, 1999).

Two groups of ten male and ten female CD1 mice received an acute high dose of the Cry2Ab2 protein by gavage. The target dose 2,198 mg/kg body weight was based on the maximum attainable Cry2Ab2 concentration of the dosing solution (estimated at 37 mg/mL) and a total dose volume of 66.6 mL/kg body weight. The limited solubility of the Cry2Ab2 protein precluded its administration as a single dose. Therefore, dosing was subdivided into two doses of 33.3 mL/kg body weight (66.6 mL/kg total) to achieve the target dose of 2,198 mg/kg. On the day of dosing (Day 0) the two individual doses of 33.3 mg/mL body weight were separated by approximately four hours. A separate control group of ten male and ten female animals received bovine serum albumin (BSA) at a dose of 2,442 mg/kg.

Following dosing, all mice were observed daily, and body weights and food consumption were measured weekly. A gross necropsy examination was performed on all animals at the time of death or the end of the study (day 14).

No mortality or treatment-related clinical observations were observed during the study. Likewise, there were no statistically significant differences in food consumption, body weight, or body weight changes. No gross pathological findings related to consumption of Cry2Ab2 protein were observed at necropsy.

Under the conditions of this test, no mortality or other evidence of toxicity was observed following Cry2Ab2 administration at the maximum attainable dose. Therefore, the acute oral LD_{50} of the Cry2Ab2 protein in mice is greater than 2,198 mg/kg body weight. This dose was determined to be the no-observed effect level (NOEL) in this study.

e) Structural similarity of the Cry1A.105 and Cry2Ab2 proteins to known toxins or other biologically active proteins

The safety assessment of proteins expressed in biotechnology-derived crops evaluates potential health effects through a comprehensive approach, which includes bioinformatic analysis of the amino acid sequence of the introduced protein to ensure the protein is not similar to toxic proteins that are known to cause adverse health effects.

The FASTA algorithm can be used to evaluate the extent of sequence alignment between a query protein sequence and a database sequence. In principle, if two proteins share sufficient linear sequence similarity and identity, they will also likely share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). The extent of structural relatedness is evaluated by visual inspection of the aligned sequence, the calculated percent identity, and *E* score. The *E* score reflects the degree of amino acid similarity between a pair of sequences and can be used to evaluate the significance of the alignment. For a pair of sequences, a very small *E* score may indicate a structurally relevant similarity. Conversely, large *E* scores are typically associated with alignments that do not represent a biologically relevant correlation. Typically, alignments between the two sequences will need to have an *E* score of less than 1×10^{-5} to be considered to have significant homology.

i) Structural similarity of Cry1A.105 to known toxins or other biologically active proteins

Potential structural identity and similarity shared between the Cry1A.105 protein and proteins in the toxin (TOXIN5) database were evaluated using the FASTA sequence alignment tool. Identified proteins were ranked according to their degree of similarity. As expected, the most significant similarity observed was to the *Bacillus thuringiensis* pesticidal crystal protein, Cry1Ac (Accession no. U89872), demonstrating 92.0% identity with 1,182 amino acids with an *E* score of zero. This alignment is expected because the Cry1A.105 protein contains a significant portion of the Cry1Ac protein, which is listed with GenBank as an insecticidal toxin and, as such, was included in the TOXIN5 database during its construction.

Potential structural similarities shared between the Cry1A.105 protein and proteins in the ALLPEPTIDES database were evaluated using the FASTA sequence alignment tool. Identified proteins were ranked according to their degree of similarity. As expected, the best similarity observed was to the pesticidal crystal protein Cry1A (GI no. 37048803), demonstrating 92.1% identity over 1,177 amino acids with an *E* score of zero. All of the remaining alignments with significant *E* scores are to Cry protein homologues except one hypothetical amino acid sequence; the hypothetically translated sequence, JMP 134, from the bacteria *Ralstonia eutropha*. Upon further examination, none of the Cry protein homologues or the *Ralstonia eutropha* bacterial sequence indicated any concern for adverse biological activity.

Therefore, results of the FASTA sequence alignments demonstrated a lack of structurally relevant similarity between the Cry1A.105 protein and any known toxic or pharmacologically active proteins relevant to human or animal health.

ii) Structural similarity of Cry2Ab2 to known toxins or other biologically active proteins

Potential structural identity and similarity shared between the Cry2Ab2 protein and proteins in the toxin (TOXIN5) database were evaluated using the FASTA sequence alignment tool. The most significant similarity observed was to the *Bacillus thuringiensis* pesticidal crystal protein (Accession no. P21254), demonstrating 100.0% identity over 632 amino acids with an *E* score of zero.

Potential structural similarities shared between the Cry2Ab2 protein and proteins in the ALLPEPTIDES database were evaluated using the FASTA sequence alignment tool. The best similarity observed was to pesticidal crystal protein Cry2Ab (GI no. 117328), demonstrating 100.0% identity over 632 amino acids with an *E* score of zero. All remaining alignments with significant *E* scores are to Cry protein homologues derived from *Bacillus thuringiensis, Clostridium bifermentans, Paenibacillus popilliae* or *Paenibacillus lentimorbus*.

Therefore, results of the FASTA sequence alignments demonstrated a lack of structurally relevant similarity between the Cry2Ab2 protein and any known toxic or pharmacologically active proteins relevant to human or animal health.

f) Source of the Cry1A.105 and Cry2Ab2 Proteins

As previously described in Section 2.2.a., Cry1A.105 is a chimeric protein consisting of domains I and II from Cry1Ab or Cry1Ac, a substantial portion of domain III from Cry1F, and the C-terminal domain from Cry1Ac. Cry1Ac and Cry1Ab are derived from *Bacillus thuringiensis* subsp. *kurstaki* and Cry1F is derived from *Bacillus thuringiensis* subsp. *aizawai*. The Cry2Ab2 protein, also present in MON 89034 is derived from *Bacillus thuringiensis* subsp. *kurstaki*. There are no known reports of allergy to *Bacillus thuringiensis* or to these Cry proteins.

g) Assessment of the potential for allergenicity of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034

The assessment of the allergenic potential of proteins compares the biochemical characteristics of the proteins in question to characteristics of known allergens. A protein is not likely to be allergenic if:

1. The protein is from a non-allergenic source;
2. The protein does not share structural similarities with known allergens based on the amino acid sequence;
3. The protein is rapidly digested in simulated digestive fluid;
4. The protein represents only a very small portion of the total protein in grain.

Section 2.5.b. and f. address points (1) and (4). Points (2) and (3) are discussed below in more detail for the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034.

i) Bioinformatic Analyses of Sequence Similarity of the Cry1A.105 and Cry2Ab2 Proteins Produced in MON 89034 to Allergens

In 2003, the Codex Alimentarius Commission published guidelines for the evaluation of the potential allergenicity of novel proteins (Codex Alimentarius, 2003). The guideline is based on the comparison of amino acid sequences between introduced proteins and allergens, where potential allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids.

It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain immunologically significant epitopes and therefore the Codex guideline also recommended that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides in otherwise unrelated proteins. An amino acid sequence may be considered to have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Metcalfe et al., 1996; Hileman et al., 2002). Using a sliding window of less than eight amino acids can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005).

- **Bioinformatic analyses of sequence similarity of the Cry1A.105 protein produced in MON 89034 to allergens**

Potential structural identity and similarity shared between the Cry1A.105 protein and proteins in the allergen database (AD6) were evaluated using the FASTA sequence alignment tool (please refer to Section 2.5.e. for a description of the FASTA sequence alignment tool). None of the proteins in the AD6 database met or exceeded the threshold of 35% identity over 80 amino acids. One low quality alignment between *Actinidia deliciosa* kiwifruit actinidin protein was identified, where a stretch of 24.2% identity over a 318 amino acid window was identified. This alignment had an *E*-score of 2.3. This *E*-score of 2.3 is not reflective of homology between Cry1A.105 and the kiwifruit protein, as *E*-scores of ~1 or larger are expected to occur for alignments between random, non-homologous sequences (Pearson, 2000). Therefore, this low quality alignment is considered not relevant from an allergenic assessment perspective. Inspection of the remaining alignments did not show any significant similarities between the Cry1A.105 protein and other allergens.

An eight-mer search was performed using an algorithm (ALLERGENSEARCH) that was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the Cry1A.105 sequence and amino acid sequences within AD6. This program compares the Cry1A.105 sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. No alignments of eight contiguous amino acid identities were detected when the Cry1A.105 protein sequence was compared to all sequences in the AD6 database.

Together, these data demonstrate that the Cry1A.105 protein does not share any relevant amino acid similarities with known allergens, gliadins, or glutenins.

- **Bioinformatic analyses of sequence similarity of the Cry2Ab2 protein produced in MON 89034 to allergens**

Potential structural identity and similarity shared between the Cry2Ab2 protein and proteins in the allergen 6 (AD6) database were evaluated using the FASTA sequence alignment tool (please refer to Section 2.5.e. for a description of the FASTA sequence alignment tool).

FASTA comparison results showed that the greatest similarity to the Cry2Ab2 protein was to the *Coprinus comatus* protein Cop c1, demonstrating only 32.7% identity over a 52 amino acid window with an *E* score of 0.89. This protein did not meet nor exceeded the threshold of 35% identity over 80 amino acids, and based on the low percent-identity and the small alignment window relative to the length of the Cry2Ab2 protein (637 amino acids), this FASTA alignment does not represent a *bona fide* homology (Doolittle, 1990). Therefore, this low quality alignment is considered not relevant from an allergenic assessment perspective. Inspection of the remaining alignments also did not show any significant similarities between the Cry2Ab2 protein and other allergens.

The pair-wise comparison of eight-mer search results showed that no immunologically relevant sequences (eight contiguous amino acid identities) were detected when the Cry2Ab2 protein sequence was compared to the sequence database.

Together these data demonstrate that the Cry2Ab2 protein is unlikely to share structurally relevant or immunologically relevant sequence similarities with known allergens, gliadins, or glutenins.

ii) Stability of the Cry1A.105 and Cry2Ab2 Proteins in Simulated Gastric Fluids

A factor that increases the likelihood of allergic oral sensitisation to proteins is the stability of the proteins to gastrointestinal digestion. Protein allergens tend to be stable to the peptic and acidic conditions of the digestive systems if they are able to reach and pass through the intestinal mucosa to elicit an allergenic response (Astwood et al., 1996; Metcalfe et al., 1996).

One aspect of this assessment includes analysis of the digestibility of the protein in a simulated gastric fluid (SGF) assay containing pepsin. A relationship between digestibility in SGF and the likelihood of being an allergen has been previously reported with a group of proteins consisting of both allergens and non-allergens (Astwood et al., 1996). Recently, the SGF assay protocol was standardised by the International Life Science Institute (ILSI) based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). This test showed that the results of *in vitro* pepsin digestion assays are reproducible when standard protocols were followed. Using these protocols, the pepsin digestion assay was used to assess the susceptibility of the Cry1A.105 and Cry2Ab2 proteins to pepsin digestion *in vitro*.

In addition to SGF, simulated intestinal fluid (SIF) is also used for *in vitro* studies to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002). SIF is an *in vitro* digestion model where proteins undergo digestion at neutral pH by a mixture of enzymes known as pancreatin. The relationship between protein allergenicity and protein stability in the *in vitro* SIF study is limited, because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case *in vivo*. *In vitro* susceptibility of Cry1A.105 and Cry2Ab2 proteins to pancreatin was assessed for digestibility in SIF according to methods described in the United States Pharmacopeia (1995).

- **Digestibility of the Cry1A.105 protein in simulated gastric fluid**

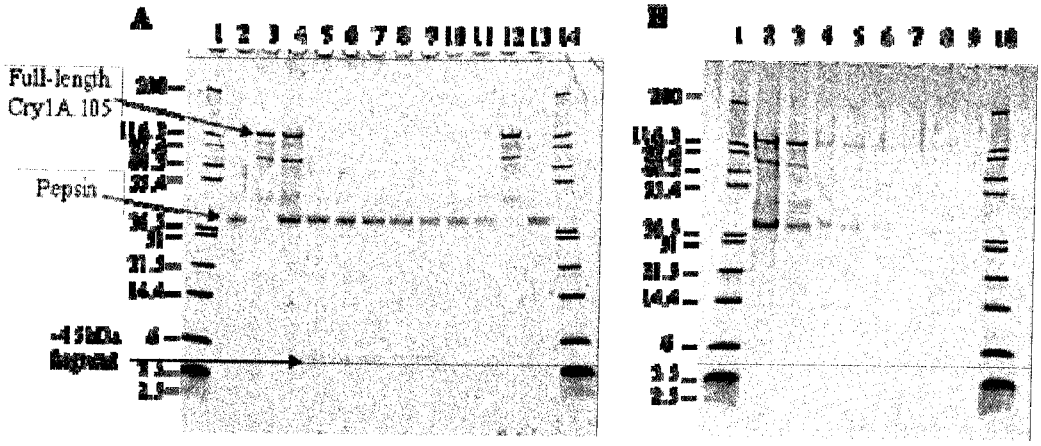
Digestibility of the Cry1A.105 protein in simulated gastric fluid (SGF) was assessed by SDS-PAGE and western blot methods. The extent of digestion of the Cry1A.105 protein was evaluated by visual analysis of stained polyacrylamide gels (**Figure 39**) or by visual analysis of developed X-ray film of western blot (**Figure 40**). A separate gel or blot was performed concurrently to determine the limit of detection (LOD) of each assay (**Figure 39**, panel B; **Figure 40**, panel B). The limit of detection of the full-length Cry1A.105 protein by Colloidal Brilliant Blue G staining was 0.005 µg or approximately 0.7% of the total Cry1A.105 protein loaded (0.005 µg divided by 0.7 µg of the loaded protein in the test). The limit of detection of the full-length Cry1A.105 protein by western blotting was 1.0 ng or approximately 5% of the total Cry1A.105 protein loaded (1 ng divided by 20 ng of the loaded protein in the test). In both methods, in addition to the full-length Cry1A.105 protein, some bands with lower molecular weight were observed. These bands represent proteolytic fragments of the Cry1A.105 protein that result from proteolysis during the purification procedure.

The gel used to assess the digestibility of the Cry1A.105 protein to pepsin (**Figure 39**, panel A) by Colloidal Brilliant Blue G staining was loaded with 0.7 µg (based on pre-digestion concentrations) for each of the digestion time points. Visual examination of the stained gel showed that the full-length Cry1A.105 protein was digested below LOD within 30 seconds of digestion in SGF (**Figure 39,** panel A, lane 5). Therefore, at least 99.3% (100% – 0.7% = 99.3%) of the full-length Cry1A.105 protein was digested within 30 seconds of incubation based on this analysis. A faint band with a molecular weight of approximately 4.5 kDa was observed at a very low level between the 30-second and 20-minute digestion time points (**Figure 39**, panel A, lanes 5-9). No protein band was visible at the 30-minute digestion time point (**Figure 39**, panel A, lane 10). Since there were only trace amounts of the ~4.5 kDa fragment present in the first 20 minutes of digestion and this fragment was undetectable in the 30 minute time point and beyond, it is unlikely to pose a human health risk.

The gel used to assess the Cry1A.105 protein *in vitro* digestibility by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that the Cry1A.105 protein was digested below the LOD within 30 seconds of incubation in SGF (**Figure 40**, panel A, lane 5). Based on the western blot LOD for the Cry1A.105 protein in SGF and the observation that no full-length protein or immunoreactive bands were observed on the western blot at the 30-second digestion time point, it was concluded

that greater than 95% (100% – 5% = 95%) of the full-length Cry1A.105 protein was digested within 30 seconds of incubation with SGFs.

The results of this study demonstrated that the full-length Cry1A.105 protein was rapidly digested after incubation in SGF. The full-length Cry1A.105 protein was digested below the LOD within 30 seconds when analysed using Colloidal Brilliant Blue G staining or by western blotting. There were no stable proteolytic degradation products, with the exception of a very faint band detected by Colloidal Brilliant Blue G staining with a MW of ~4.5 kDa; this band was not observed in the 30-minute time point or beyond. Overall, the results for digestibility of Cry1A.105 are consistent with proteins with demonstrated safety. The fact that Cry1A.105 is readily digestible in simulated gastric fluid makes it unlikely to be a food allergen.



Lane assignment for Panel A

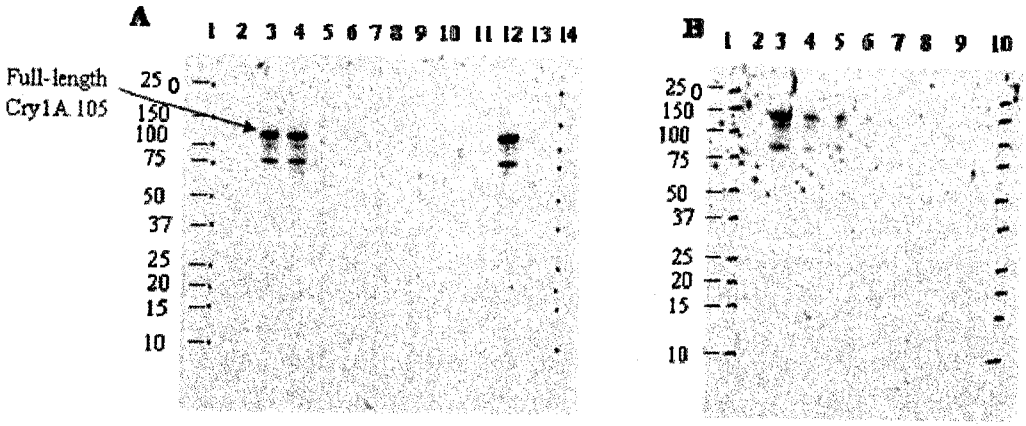
Lane	Sample	Incubation Time (min)
1	MW marker	—
2	N0, SGF only	0
3	P0, protein only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein +SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	MW marker	—

Lane assignment for Panel B

Lane	Sample	Amount (µg)
1	MW marker	—
2	T0, protein+SGF	0.7
3	T0, protein+SGF	0.35
4	T0, protein+SGF	0.1
5	T0, protein+SGF	0.05
6	T0, protein+SGF	0.02
7	T0, protein+SGF	0.01
8	T0, protein+SGF	0.005
9	T0, protein+SGF	0.0025
10	MW marker	—

Figure 39. Colloidal Brilliant Blue G stained SDS-gels of Cry1A.105 protein digestion in SGF

Panel A corresponds to Cry1A.105 protein digestion in SGF. Based on pre-digestion protein concentrations, 0.7 µg (total Cry1A.105 protein) was loaded in lanes containing Cry1A.105 protein. The incubation times are indicated. Panel B corresponds to the limit of detection (LOD) of Cry1A.105 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.



Lane assignment for Panel A

Lane	Sample	Incubation Time (min)
1	MW marker	—
2	N0, SGF only	0
3	P0, protein only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	MW marker	—

Lane assignment for Panel B

Lane	Sample	Amount (ng)
1	MW marker	—
2	T0, protein+SGF	7
3	T0, protein+SGF	3.5
4	T0, protein+SGF	2
5	T0, protein+SGF	1
6	T0, protein+SGF	0.5
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	MW marker	—

Figure 40. Western blot analysis of Cry1A.105 protein digestion in SGF

Panel A corresponds to Cry1A.105 protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in lanes containing Cry1A.105 protein. Panel B corresponds to the limit of detection (LOD) of the Cry1A.105 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.

- **Digestibility of the Cry1A.105 protein in simulated intestinal fluid**

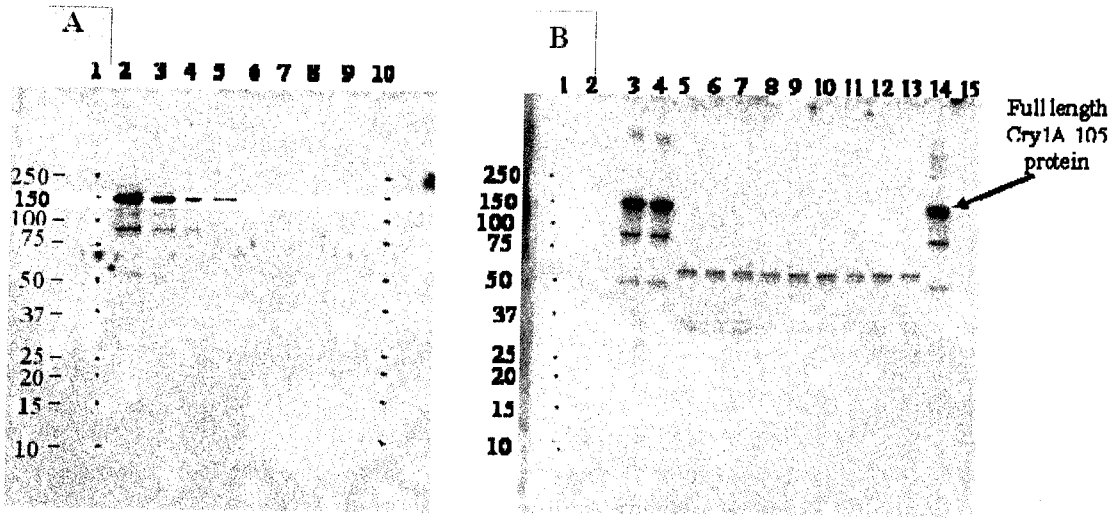
The digestibility of the Cry1A.105 protein in simulated intestinal fluid (SIF) was assessed where SIF contained a mixture of enzymes called pancreatin.

The digestion of the Cry1A.105 protein was evaluated by western blot method (**Figure 41**). A western blot to determine the LOD (**Figure 41**, panel A) of the Cry1A.105 protein was performed concurrently with the western blot used to assess the Cry1A.105 protein digestibility in SIF (**Figure 41**, panel B). The LOD was estimated to be 0.1 ng, which represented 0.5% of the total protein loaded in this experiment (0.1 ng divided by 20 ng of loaded protein).

The gel to assess the Cry1A.105 protein SIF digestibility by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that the full-length Cry1A.105 protein was digested below the LOD within 5 minutes of incubation in SIF (**Figure 41**, panel B, lane 5). Therefore, at least 99.5% (100% - 0.5% = 99.5%) of the full-length Cry1A.105 protein was broken down within 5 minutes. Proteolytic fragments with approximate molecular weight of ~60, 32 and 30 kDa were observed at the 5 minute digestion time point and were stable for the various times up to 24 hours of digestion in SIF.

The results of this study demonstrate that the full-length Cry1A.105 protein was digested within 5 min of incubation in SIF, yielding fragments with molecular weights of approximately 60, 32, and 30 kDa. At least 99.5% of the full-length Cry1A.105 protein was digested in SIF within 5 minutes. The major proteolytic fragment at approximately 60 kDa migrated as a doublet, which represents the tryptic core of the Cry1A.105 protein, was observed for up to 24 hours (the longest time point tested). These results are consistent with observations for other Cry proteins with demonstrated safety.

Overall, the results for digestibility of Cry1A.105 are consistent with proteins with demonstrated safety. The fact that Cry1A.105 is readily digestible in simulated gastric fluid makes it unlikely be a food allergen.



Lane assignment for Panel A

Lane	Sample	Amount (ng)
1	Molecular weight marker	—
2	T0, protein+SIF	10
3	T0, protein+SIF	5
4	T0, protein+SIF	2
5	T0, protein+SIF	1
6	T0, protein+SIF	0.5
7	T0, protein+SIF	0.2
8	T0, protein+SIF	0.1
9	T0, protein+SIF	0.05
10	Molecular weight marker	—

Lane assignment for Panel B

Lane	Sample	Digestion Time
1	Molecular weight marker	—
2	N0, SIF only	0
3	P0, protein only	0
4	T0, protein+SIF	0
5	T1, protein+SIF	5 min
6	T2, protein+SIF	15 min
7	T3, protein+SIF	30 min
8	T4, protein+SIF	1 h
9	T5, protein+SIF	2 h
10	T6, protein+SIF	4 h
11	T7, protein+SIF	8 h
12	T8, protein+SIF	12 h
13	T9, protein+SIF	24 h
14	P9, protein only	24 h
15	N9, SIF only	24 h

Figure 41. Western blot analysis of Cry1A.105 protein digestion in SIF
Panel A corresponds to the limit of detection of Cry1A.105 protein. Panel B corresponds to Cry1A.105 protein digestion in SIF. Based on the pre-digestion protein concentration, 20 ng (total protein) was loaded in lanes containing Cry1A.105 protein. The digestion times are indicated. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel.

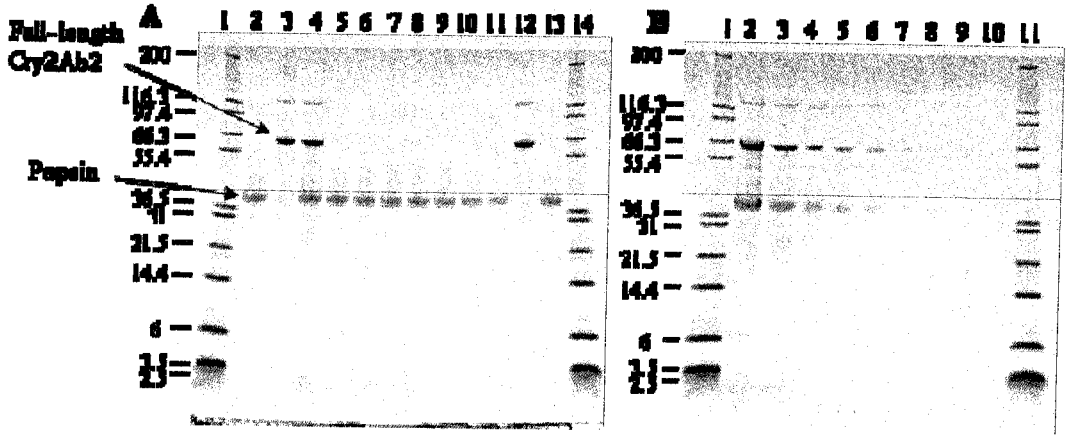
- **Digestibility of the Cry2Ab2 protein in simulated gastric fluid**

Digestibility of the Cry2Ab2 protein in SGF was assessed by SDS-PAGE and western blot methods. The extent of digestion of the Cry2Ab2 protein was evaluated by visual analysis of stained polyacrylamide gels (**Figure 42**) or by visual analysis of developed X-ray film of western blot (**Figure 43**). A separate gel or blot was performed concurrently to determine the limit of detection (LOD) of each assay (**Figure 42**, panel B; **Figure 43**, panel B). The limit of detection of the full-length Cry2Ab2 protein by Colloidal Brilliant Blue G staining was 0.005 µg or approximately 0.6% of the total Cry2Ab2 protein loaded (0.005 µg divided by 0.8 µg of the loaded protein in the test). The limit of detection of the full-length Cry2Ab2 protein by western blotting was 0.2 ng or approximately 1% of the total Cry2Ab2 protein loaded (0.2 ng divided by 20 ng of the loaded protein in the test).

Digestibility of the Cry2Ab2 protein in SGF assessed using stained SDS-polyacrylamide gels showed that the full-length Cry2Ab2 protein was rapidly digested. At least 99.4% of the full-length Cry2Ab2 protein was digested within 30 seconds when analysed using Colloidal Brilliant Blue G stained polyacrylamide gels (**Figure 42**). A very faint band with molecular weight of ~5 kDa was observed at the 30-second digestion time point. No Cry2Ab2 bands were visible at the 2-minute digestion time point (**Figure 42**, panel A, lane 6).

The extent of digestion of the Cry2Ab2 protein was also evaluated by western blot method (**Figure 43**). At least 99% of the Cry2Ab2 protein was digested in SGF within 30 seconds when analysed using western blot analysis (**Figure 43**, panel A). No stable proteolytic bands were observed at any time points by western blot analysis.

The results of this study demonstrated that Cry2Ab2 protein was rapidly digested after incubation in SGF. The Cry2Ab2 protein was digested below the LOD within 30 seconds when analysed using SDS-PAGE by Colloidal Brilliant Blue G staining or by western blotting. There were no stable proteolytic degradation products detected. The fact that Cry2Ab2 is readily digestible in simulated gastric fluid makes it unlikely to be a food allergen.



Lane assignment for Panel A

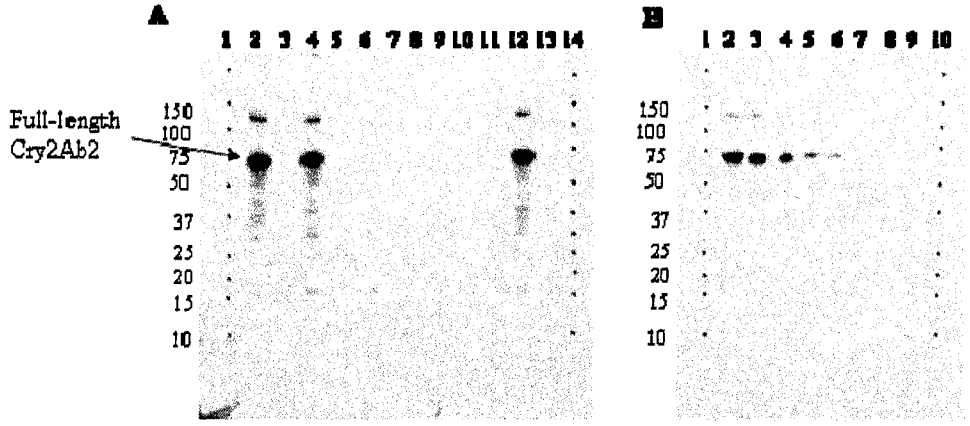
Lane	Sample	Incubation Time (min)
1	Molecular weight marker	n/a
2	N0, SGF only	0
3	P0, protein only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	Molecular weight marker	n/a

Lane assignment for Panel B

Lane	Sample	Amount (µg)
1	Molecular weight marker	n/a
2	T0, protein+SGF	1
3	T0, protein+SGF	0.5
4	T0, protein+SGF	0.2
5	T0, protein+SGF	0.1
6	T0, protein+SGF	0.05
7	T0, protein+SGF	0.02
8	T0, protein+SGF	0.01
9	T0, protein+SGF	0.005
10	T0, protein+SGF	0.0025
11	Molecular weight marker	n/a

Figure 42. Colloidal Brilliant Blue G stained SDS-PAGE gels of Cry2Ab2 protein digestion in SGF

Panel A corresponds to Cry2Ab2 protein digestion in SGF. Based on the pre-digestion protein concentration, ~0.8 µg (total protein) was loaded in lanes containing Cry2Ab2 protein. Panel B corresponds to the limit of detection (LOD) of Cry2Ab2 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.



Lane assignment for Panel A

Lane	Sample	Incubation Time (min)
1	Molecular weight marker	n/a
2	P0, protein only	0
3	N0, SGF only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	Molecular weight marker	n/a

Lane assignment for Panel B

Lane	Sample	Amount (ng)
1	Molecular weight marker	n/a
2	T0, protein+SGF	10
3	T0, protein+SGF	5
4	T0, protein+SGF	2
5	T0, protein+SGF	1
6	T0, protein+SGF	0.5
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	Molecular weight marker	n/a

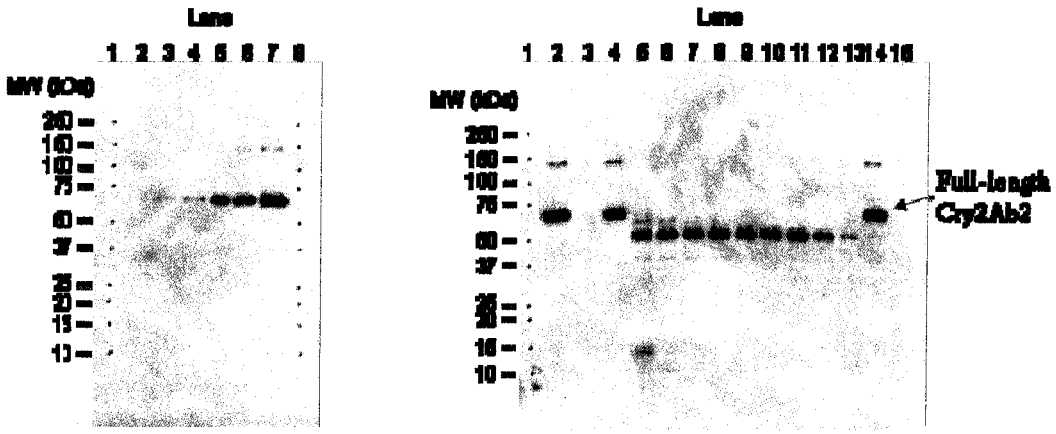
Figure 43. Western blot analysis of Cry2Ab2 protein digestion in SGF
Panel A corresponds to Cry2Ab2 protein digestion in SGF. Based on the pre-digestion protein concentration, 20 ng (total protein) was loaded in lanes containing Cry2Ab2 protein. Panel B corresponds to the limit of detection of the Cry2Ab2 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.

- **Digestibility of the Cry2Ab2 protein in simulated intestinal fluid**

The *in vitro* digestibility of the Cry2Ab2 protein in SIF was assessed by western blot analysis. A western blot to determine the LOD (**Figure 44**, panel A) of the Cry2Ab2 protein was run concurrently with the western blot used to assess the Cry2Ab2 protein digestibility in SIF (**Figure 44**, panel B). The LOD was determined by the lowest amount of Cry2Ab2 protein observed on the X-ray film under visual inspection. The LOD was estimated to be 0.5 ng, which represents 2.5% of the total protein (0.5 ng divided by 20 ng of loaded protein in the test).

The gel used to assess the Cry2Ab2 protein digestibility by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the incubation time points. Western blot analysis demonstrated that a band corresponding to the full-length Cry2Ab2 protein was digested below the LOD within 15 minutes of incubation in SIF (**Figure 44**, panel B, lane 6). Therefore, at least 97.5% (100% - 2.5% = 97.5%) of the full-length Cry2Ab2 protein was digested within 15 minutes. Proteolytic bands with approximate molecular weight of 60, 55, 50, 40, 12 and 10 kDa were observed at the 5-minute time point. Several new bands with molecular weights smaller than 50 kDa were detectable beginning at the 4-hour digestion time point. These bands, which were transient in nature and displayed a weak immunoreactive signal, were detectable at the 24-hour incubation time point.

Overall, the results for digestibility of Cry2Ab2 are consistent with other Cry proteins with demonstrated safety. The fact that Cry2ABb2 is readily digestible in simulated gastric fluid makes it unlikely be a food allergen



Lane assignment for Panel A

Lane	Sample	Amount (ng)
1	Molecular weight marker	—
2	T0, protein+SIF	10
3	T0, protein+SIF	5
4	T0, protein+SIF	2
5	T0, protein+SIF	1
6	T0, protein+SIF	0.5
7	T0, protein+SIF	0.2
8	T0, protein+SIF	0.1
9	T0, protein+SIF	0.05
10	Molecular weight marker	—

Lane assignment for Panel B

Lane	Sample	Digestion Time
1	Molecular weight marker	—
2	N0, SIF only	0
3	P0, protein only	0
4	T0, protein+SIF	0
5	T1, protein+SIF	5 min
6	T2, protein+SIF	15 min
7	T3, protein+SIF	30 min
8	T4, protein+SIF	1 h
9	T5, protein+SIF	2 h
10	T6, protein+SIF	4 h
11	T7, protein+SIF	8 h
12	T8, protein+SIF	12 h
13	T9, protein+SIF	24 h
14	P9, protein only	24 h
15	N9, SIF only	24 h

Figure 44. Western blot analysis of Cry2Ab2 protein digestion in SIF

Panel A corresponds to the LOD of Cry2Ab2 protein (5 min exposure). Panel B corresponds to Cry2Ab2 protein digestion in SIF (5 min exposure). Based on the pre-digestion concentration, 20 ng (total protein) was loaded in lanes containing Cry2Ab2 protein. The incubation times are indicated. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel.

2.6 Comparative Analysis

Maize is widely used for food and feed purposes, and it is intended that MON 89034 will be utilised in the same manner and for the same uses as conventional field maize. To assess whether the introduction or expression of the insect protection traits in MON 89034 caused any unintended effects, compositional analyses were conducted on the maize grain and forage of MON 89034. These assessments are conducted according to the principles of comparative risk assessment or substantial equivalence, which has been adopted by a number of international organisations including the World Health Organisation (WHO), United Nations Food and Agricultural Organisation (FAO), and the Organisation for Economic Cooperation and Development (OECD). According to this principle, the food or feed from a biotechnology-derived crop are compared with its conventional counterpart as an initial step in the safety assessment process. The goal is to determine whether the nutrient and anti-nutrient levels in grain and forage tissues from the biotechnology-derived crop are comparable to those of the conventional crop.

a) Levels of Significant Nutrients, Antinutrients, and Other Components in Maize Grain and Forage

Compositional analyses were conducted to assess whether the nutrient, antinutrient and secondary metabolite levels in the grain and forage tissues derived from MON 89034 are comparable to those in the conventional control, LH198 x LH172. The conventional control has background genetics similar to MON 89034, but does not contain the *cry1A.105* and *cry2Ab2* genes. Additional conventional maize hybrids currently in the marketplace were also included in the analysis to establish a range of natural variability for each analyte, which is defined by a 99% tolerance interval for that particular analyte (Ridley et al., 2002). Results of the comparisons indicate that MON 89034 is compositionally and nutritionally equivalent to conventional maize hybrids currently in commerce.

Grain and forage tissues of MON 89034 and control maize were harvested from plants grown at each of five field sites during 2004. The field sites were located in regions of the U.S. that are conducive to the growth of maize, and representative of commercial maize production. Seed was planted in a randomised complete block design with three replicates per block. In addition, 15 conventional maize hybrids were also included as references by growing three different hybrids at each of five sites for a total of 15 references. The 15 conventional maize reference hybrids were included to provide data for the development of a 99% tolerance interval for each component analysed. For each compositional component, a 99% tolerance interval was calculated. This interval is expected to contain, with 95% confidence, 99% of the values obtained from the population of commercial references. It is important to establish the 99% tolerance interval from representative conventional maize hybrids for each of the analytes, because such data illustrate the compositional variability that naturally occurs in commercially grown varieties. By comparison to the 99% tolerance interval, any statistically significant difference between MON 89034 and the control may be put into perspective, and can be assessed for biological relevance in the context of the natural variability in maize.

The compositional analyses were conducted on a total of 77 components - nine in forage and 68 in grain. Components were selected based on recommendations of the OECD (OECD, 2002). Compositional analyses of the forage samples included proximates (protein, fat, ash, and moisture), acid detergent fibre (ADF), neutral detergent fibre (NDF), minerals (calcium and phosphorus), and carbohydrates by calculation. Compositional analyses of the grain samples included proximates (protein, fat, ash, and moisture), ADF, NDF, total dietary fibre (TDF), amino acids, fatty acids (C8-C22), vitamins (B1, B2, B6, E, niacin, and folic acid), antinutrients (phytic acid and raffinose), secondary metabolites (furfural, ferulic acid, and p-coumaric acid), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and carbohydrates by calculation. Methods for analysis were based on internationally-recognised procedures and literature publications.

Statistical analyses of the compositional data were conducted using a mixed model analysis of variance. Each individual analyte for MON 89034 was compared to that of the conventional control, for the combination of all five sites (i.e., the combined-site) and for each individual site. The statistical significance was defined at the level of $p<0.05$. Of the 77 components analysed, 16 components had greater than 50% of the analytical values that were below the limit of quantitation, and therefore, were not included in the statistical analyses.

Statistical analyses of the remaining 61 components (77 minus the 16) were conducted for comparison of MON 89034 with control maize. The overall data set was examined for evidence of biologically relevant changes. Based on this evaluation and the results of statistical analyses, analytes for which the levels were not statistically different were deemed to be present at equivalent levels between MON 89034 and the control. Analyses using data from the combined sites indicated that there were no statistical differences in the levels of 95% of the analytes (58 of the 61). Statistical analyses for the combined-site data are presented in **Table 11** for forage and **Tables 12** to **17** for grain. Analyses using data from the five single sites indicated that there were no statistically significant differences in analytes for 261 of 305 comparisons made between MON 89034 and the control.

- **Assessment of levels of nutrients in maize forage and grain**

For the combined-site analyses, statistical differences between MON 89034 and control maize were observed for three analytes, which included phosphorus in forage, and 18:0 stearic and 20:0 arachidic acids in grain (**Table 18**). The differences observed are generally small (3.4 – 19.2%) considering the natural variability, and the mean levels and ranges of MON 89034 are well within the 99% tolerance intervals for commercial maize. The mean levels and ranges of phosphorus in forage, and 18:0 stearic and 20:0 arachidic acids in grain, were also within the ranges in the International Life Sciences Institute Crop Composition Database (ILSI-CCD, 2006), as well as within published literature ranges. Therefore, it is concluded that MON 89034 and control maize are compositionally equivalent based on analyses of the combined-site data. The reported ILSI and published literature ranges for the analytical components present in maize are summarised in **Table 19.**

The reproducibility and trends at individual sites were also examined, and comparisons made to conventional maize hybrids using the 99% tolerance intervals. Of the 44 statistical differences observed in the individual site analyses, 33 were only observed at one site. There were no consistent trends and, except in two cases, the mean and ranges of the analytes in MON 89034 were well within the 99% tolerance interval for conventional maize. The mean levels and ranges for calcium and methionine in grain were slightly outside the 99% tolerance interval but within the ILSI database. It is concluded that these differences are not biologically significant. Of the remaining 11 differences observed at more than one site, there were no analytes that were consistently and statistically different across five or four sites. In addition, there were no analytes that showed statistically significant differences in three sites that had not been previously observed (i.e., 20:0 arachidic acid) to be different in the combined-site analysis. Statistically significant differences were observed in as many as two sites for three analytes (carbohydrates, copper, iron), which were not previously found to be different (i.e., 18:0 stearic acid) in the combined-site analysis. For carbohydrates and iron, the observed differences from the control were small and lower at one site and higher at the other site (**Table 18**). As there is no evidence of any reproducibility across sites, it is concluded that the statistical differences are not biologically relevant for carbohydrates and iron. For copper, the differences for MON 89034 were higher than the control at both sites (**Table 18**). The observed differences are small in magnitude, and the mean levels and ranges of MON 89034 are well within the 99% tolerance interval. Therefore, it is concluded that the statistical differences for copper are not biologically relevant.

Based on the data and information presented above, it was concluded that maize grain and forage derived from MON 89034 are compositionally and nutritionally equivalent to those of conventional maize. The few statistical differences between MON 89034 and control maize likely reflect the natural variability of the components since the mean levels of analytes for MON 89034 are well within the 99% tolerance intervals for conventional maize, and/or within the ranges in the ILSI database (ILSI-CCD, 2006) and the scientific literature.

b) Assessment of Levels of Anti-nutrient and key secondary metabolites in maize forage and grain

A description of the anti-nutrients and key secondary metabolites present in maize is provided in the OECD consensus document on compositional considerations (OECD, 2002). The anti-nutrients include phytic acid, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), raffinose, and trypsin and chymotrypsin inhibitors. The secondary metabolites include furfural, ferulic acid, and p-coumaric acid. The OECD does not recommend analysis of DIMBOA due to the variable levels found across maize hybrids. Similarly, analysis of trypsin and chymotrypsin inhibitors is not recommended because they occur at low levels in maize and are not considered nutritionally significant. According to the OECD (2002), "in considering the anti-nutrients and natural toxins in maize, only phytic acid is significant to the animal feed". The anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid and p-coumaric acid were analysed in the grain of MON 89034 and compared to those of control maize. Furfural was excluded from further consideration since the levels present in MON 89034 and control maize were at or below the limit of quantitation of the assay.

The overall data set was examined for evidence of biologically relevant changes. In addition, statistical analyses were applied to assess significant differences of each analyte at $p<0.05$. As described in **Section 2.6.a.**, 58 of 61 comparisons in the combined-site analysis and 261 of 305 comparisons in the single site analysis showed no statistically significant differences between MON 89034 and control maize. The few observed statistical differences were not considered biologically relevant since these differences were not reproducible across sites and no consistent trends were observed. Furthermore, the mean levels and ranges of MON 89034 analytes were within the 99% tolerance intervals, and/or within the ILSI database and literature ranges. The statistical summaries for the anti-nutrients are described under the grain analyses, where the combined-site data are presented in **Table 17**. The analytes that are statistically different between MON 89034 and control maize are presented in **Table 18**. The ILSI and literature ranges for maize are provided in **Table 19**.

Phytic acid is considered an important anti-nutrient for animals, especially non-ruminants, since it can significantly reduce the bioavailability of phosphorus in maize tissues. Feed formulators add the enzyme phytase to swine and poultry diets to improve the utilisation of phosphorus. Ruminants are more efficient in utilising phytic acid since microbes in the rumen produce phytase that breaks down phytate and releases phosphorus (Ensminger et al., 1990). Compositional analyses of the grain indicated that phytic acid was present at similar levels in MON 89034 and control maize, and no statistical differences were observed for all comparisons.

Raffinose is a low molecular weight carbohydrate present in maize grain that is considered an anti-nutrient due to the gas production and resulting flatulence caused by consumption (Maynard et al., 1979). Composition analyses of the grain indicated that raffinose was present at similar levels in MON 89034 and control maize, and no statistical differences were observed for all comparisons.

The phenolic acids, ferulic acid and p-coumaric acid, are structural and functional components of plant cells (Kroon and Williamson, 1999). They are found in vegetables, fruit and cereals and act as natural pesticides against insect and fungal pests. Composition analyses of the grain indicated that ferulic acid was present at similar levels in MON 89034 and control maize, and no statistical differences were observed for all comparisons. A statistical significant difference was observed for p-coumaric acid at one site but not at the other sites. Since there was no trend, the statistically significant difference for p-coumaric acid is not considered reproducible and hence not biologically significant.

Based on these results, it is concluded that the levels of anti-nutrients and key secondary metabolites (as defined by the OECD) in MON 89034 are comparable to those found in conventional maize.

Table 11. Comparison of Proximates, Fibre, and Mineral Content in Forage from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Fibre						
Acid Detergent Fibre (% DW)	28.95 ± 1.69 (22.60 - 35.85)	27.26 ± 1.69 (19.93 - 35.59)	1.69 ± 1.18 (-6.22 - 10.45)	-0.81,4.19	0.170	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fibre (% DW)	39.69 ± 1.32 (33.99 - 46.82)	37.60 ± 1.32 (31.44 - 43.96)	2.09 ± 1.40 (-3.47 - 7.47)	-0.88,5.05	0.155	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.20 ± 0.019 (0.16 - 0.24)	0.19 ± 0.019 (0.13 - 0.28)	0.0066 ± 0.011 (-0.036 - 0.063)	-0.017,0.031	0.569	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.25 ± 0.011 (0.22 - 0.32)	0.21 ± 0.011 (0.15 - 0.25)	0.040 ± 0.014 (-0.0019 - 0.13)	0.011,0.069	0.010	(0.14 - 0.25) [0.071,0.32]
Proximate						
Ash (% DW)	3.70 ± 0.27 (2.51 - 4.67)	3.90 ± 0.27 (2.59 - 5.10)	-0.20 ± 0.21 (-1.72 - 0.97)	-0.65,0.25	0.356	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	86.90 ± 0.43 (84.93 - 89.13)	86.69 ± 0.43 (84.36 - 89.57)	0.21 ± 0.53 (-4.23 - 4.41)	-0.91,1.33	0.697	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	72.20 ± 1.35 (68.50 - 75.40)	71.53 ± 1.35 (65.90 - 76.80)	0.67 ± 0.52 (-3.50 - 4.20)	-0.44,1.77	0.220	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	7.82 ± 0.27 (6.34 - 8.98)	7.70 ± 0.27 (6.06 - 8.87)	0.13 ± 0.26 (-2.32 - 2.35)	-0.43,0.68	0.635	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	1.57 ± 0.24 (0.63 - 3.17)	1.71 ± 0.24 (0.77 - 2.91)	-0.13 ± 0.23 (-2.28 - 1.95)	-0.59,0.32	0.558	(0.58 - 3.11) [0,4.54]

[1] DW = dry weight; FW = fresh weight; S.E. = standard error; CI = confidence interval.
[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 12. Comparison of the Amino Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Int.[2]]
			Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	
Amino Acid (% DW)						
Alanine (% DW)	0.77 ± 0.039 (0.64 - 0.89)	0.78 ± 0.039 (0.67 - 0.89)	-0.0070 ± 0.019 (-0.13 - 0.089)	-0.046,0.032	0.709	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.48 ± 0.013 (0.38 - 0.52)	0.47 ± 0.013 (0.41 - 0.51)	0.011 ± 0.012 (-0.090 - 0.062)	-0.014,0.036	0.361	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.68 ± 0.029 (0.56 - 0.78)	0.67 ± 0.029 (0.60 - 0.76)	0.0038 ± 0.015 (-0.11 - 0.078)	-0.028,0.036	0.804	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.23 ± 0.0057 (0.20 - 0.26)	0.23 ± 0.0057 (0.21 - 0.25)	0.0023 ± 0.0038 (-0.022 - 0.023)	-0.0057,0.010	0.554	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	1.97 ± 0.097 (1.63 - 2.29)	1.99 ± 0.097 (1.70 - 2.26)	-0.012 ± 0.049 (-0.33 - 0.24)	-0.11,0.091	0.809	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.38 ± 0.0087 (0.32 - 0.41)	0.38 ± 0.0087 (0.36 - 0.41)	0.0042 ± 0.0071 (-0.067 - 0.035)	-0.011,0.019	0.566	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.31 ± 0.011 (0.25 - 0.35)	0.31 ± 0.011 (0.28 - 0.34)	0.0027 ± 0.0055 (-0.050 - 0.030)	-0.0090,0.014	0.632	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.36 ± 0.018 (0.30 - 0.43)	0.36 ± 0.018 (0.30 - 0.42)	-0.00003 ± 0.0088 (-0.056 - 0.041)	-0.019,0.019	0.997	(0.32 - 0.45) [0.23,0.51]

Table 12 (cont). Comparison of the Amino Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Int.[2]]
			Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	
Amino Acid (% DW)						
Leucine (% DW)	1.31 ± 0.077 (1.09 - 1.57)	1.32 ± 0.077 (1.08 - 1.55)	-0.014 ± 0.036 (-0.21 - 0.16)	-0.089,0.062	0.700	(1.14 - 1.68) [0.77,1.92]
Lysine (% DW)	0.33 ± 0.0097 (0.26 - 0.36)	0.32 ± 0.0097 (0.29 - 0.36)	0.0088 ± 0.0078 (-0.056 - 0.033)	-0.0077,0.025	0.273	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.23 ± 0.0064 (0.20 - 0.27)	0.22 ± 0.0064 (0.20 - 0.24)	0.0038 ± 0.0047 (-0.017 - 0.028)	-0.0061,0.014	0.427	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.51 ± 0.028 (0.43 - 0.61)	0.52 ± 0.028 (0.43 - 0.60)	-0.0012 ± 0.013 (-0.080 - 0.067)	-0.029,0.026	0.925	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	0.93 ± 0.030 (0.79 - 1.05)	0.93 ± 0.030 (0.83 - 1.01)	0.0034 ± 0.019 (-0.15 - 0.10)	-0.037,0.044	0.861	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.52 ± 0.022 (0.44 - 0.61)	0.52 ± 0.022 (0.46 - 0.60)	-0.0046 ± 0.012 (-0.087 - 0.058)	-0.030,0.021	0.703	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.33 ± 0.010 (0.27 - 0.37)	0.33 ± 0.010 (0.29 - 0.36)	0.00063 ± 0.0074 (-0.052 - 0.039)	-0.015,0.016	0.933	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.056 ± 0.0018 (0.048 - 0.064)	0.056 ± 0.0018 (0.045 - 0.063)	0.00031 ± 0.0013 (-0.0055 - 0.0072)	-0.0025,0.0031	0.817	(0.043 - 0.059) [0.032,0.072]

Table 12 (cont). Comparison of the Amino Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Amino Acid (% DW)						
Tyrosine (% DW)	0.37 ± 0.015 (0.22 - 0.43)	0.36 ± 0.015 (0.24 - 0.42)	0.0088 ± 0.016 (-0.21 - 0.14)	-0.026,0.043	0.596	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.49 ± 0.020 (0.40 - 0.55)	0.49 ± 0.020 (0.43 - 0.55)	0.0034 ± 0.010 (-0.084 - 0.055)	-0.019,0.026	0.748	(0.42 - 0.55) [0.35,0.62]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 13. Comparison of the Fatty Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Int.[2]]
			Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.19 ± 0.060 (8.98 - 9.46)	9.12 ± 0.060 (8.91 - 9.34)	0.071 ± 0.049 (-0.14 - 0.33)	-0.034,0.18	0.171	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.13 ± 0.0058 (0.11 - 0.14)	0.12 ± 0.0058 (0.048 - 0.14)	0.0022 ± 0.0054 (-0.012 - 0.079)	-0.0093,0.014	0.696	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.89 ± 0.021 (1.79 - 2.03)	1.82 ± 0.021 (1.76 - 1.87)	0.072 ± 0.021 (-0.055 - 0.18)	0.028,0.12	0.002	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	24.96 ± 0.34 (23.38 - 25.75)	24.84 ± 0.34 (23.62 - 26.66)	0.12 ± 0.20 (-1.48 - 1.15)	-0.32,0.55	0.574	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	61.82 ± 0.40 (60.85 - 63.61)	62.07 ± 0.40 (60.51 - 63.41)	-0.25 ± 0.23 (-1.62 - 1.24)	-0.73,0.24	0.292	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.19 ± 0.027 (1.12 - 1.23)	1.22 ± 0.027 (1.15 - 1.43)	-0.028 ± 0.016 (-0.23 - 0.036)	-0.063,0.0061	0.099	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.39 ± 0.0062 (0.36 - 0.42)	0.38 ± 0.0062 (0.36 - 0.40)	0.013 ± 0.0031 (-0.019 - 0.032)	0.0063,0.019	<0.001	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.28 ± 0.0040 (0.26 - 0.29)	0.28 ± 0.0040 (0.25 - 0.29)	0 ± 0.0024 (-0.014 - 0.011)	-0.0051,0.0051	0.999	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.16 ± 0.0050 (0.13 - 0.20)	0.15 ± 0.0050 (0.13 - 0.18)	0.0027 ± 0.0062 (-0.019 - 0.029)	-0.010,0.016	0.665	(0.12 - 0.19) [0.081.0.23]

[1] FA = fatty acid; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 14. Comparison of the Mineral Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Mineral						
Calcium (% DW)	0.0050 ± 0.00034 (0.0038 - 0.0066)	0.0049 ± 0.00034 (0.0040 - 0.0059)	0.00016 ± 0.00011 (-0.00027 - 0.00090)	-0.00008,0.00040	0.180	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	1.74 ± 0.38 (1.33 - 2.38)	2.07 ± 0.37 (1.26 - 4.54)	-0.33 ± 0.53 (-2.96 - 0.78)	-1.45,0.79	0.547	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	21.40 ± 1.00 (19.23 - 25.23)	22.20 ± 0.99 (19.03 - 28.26)	-0.80 ± 0.67 (-6.50 - 5.90)	-2.22,0.62	0.250	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.12 ± 0.0043 (0.10 - 0.14)	0.12 ± 0.0043 (0.11 - 0.14)	-0.00028 ± 0.0021 (-0.018 - 0.011)	-0.0047,0.0041	0.893	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	6.79 ± 0.29 (5.43 - 9.32)	6.51 ± 0.29 (5.57 - 8.00)	0.28 ± 0.21 (-1.54 - 2.36)	-0.18,0.73	0.213	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.33 ± 0.0095 (0.27 - 0.36)	0.33 ± 0.0095 (0.29 - 0.36)	0.00039 ± 0.0043 (-0.038 - 0.026)	-0.0087,0.0095	0.929	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.36 ± 0.0065 (0.32 - 0.40)	0.36 ± 0.0065 (0.34 - 0.40)	0.0032 ± 0.0042 (-0.030 - 0.035)	-0.0052,0.012	0.450	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	22.05 ± 1.14 (18.91 - 26.89)	21.91 ± 1.14 (18.81 - 26.04)	0.14 ± 0.51 (-3.37 - 3.19)	-0.94,1.22	0.788	(16.72 - 34.04) [7.16,38.55]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.
[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 15. Comparison of the Proximates and Fibre Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E. (Range)	Control Mean ± S.E.[1] (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Proximate						
Ash (% DW)	1.41 ± 0.036 (1.25 - 1.56)	1.39 ± 0.036 (1.28 - 1.51)	0.014 ± 0.041 (-0.11 - 0.13)	-0.072,0.10	0.734	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	84.85 ± 0.42 (83.29 - 86.52)	84.96 ± 0.42 (83.58 - 86.22)	-0.11 ± 0.18 (-1.42 - 0.84)	-0.50,0.28	0.562	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	9.52 ± 0.77 (7.89 - 12.80)	9.50 ± 0.77 (7.86 - 13.10)	0.021 ± 0.22 (-1.00 - 0.87)	-0.44,0.48	0.923	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	10.43 ± 0.42 (8.54 - 11.98)	10.36 ± 0.42 (9.22 - 11.52)	0.070 ± 0.19 (-1.26 - 1.28)	-0.34,0.48	0.725	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.32 ± 0.069 (3.05 - 3.89)	3.29 ± 0.069 (3.05 - 3.75)	0.025 ± 0.089 (-0.50 - 0.29)	-0.16,0.21	0.784	(2.66 - 3.71) [2.20,4.55]
Fibre						
Acid Detergent Fibre (% DW)	5.48 ± 0.19 (3.82 - 7.24)	5.27 ± 0.19 (4.17 - 7.00)	0.21 ± 0.25 (-3.18 - 3.07)	-0.30,0.72	0.410	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fibre (% DW)	10.06 ± 0.37 (8.59 - 12.08)	9.75 ± 0.37 (8.48 - 11.75)	0.31 ± 0.34 (-2.26 - 2.05)	-0.41,1.03	0.370	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fibre (% DW)	15.17 ± 0.47 (13.39 - 17.02)	14.67 ± 0.47 (12.82 - 17.62)	0.50 ± 0.54 (-3.61 - 4.20)	-0.66,1.65	0.375	(12.99 - 18.03) [9.20,20.27]

[1] DW = dry weight; FW = fresh weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 16. Comparison of the Vitamin Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Vitamin						
Folic Acid (mg/kg DW)	0.35 ± 0.037 (0.26 - 0.48)	0.36 ± 0.037 (0.23 - 0.53)	-0.0080 ± 0.022 (-0.11 - 0.11)	-0.054,0.038	0.717	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	30.08 ± 1.11 (25.72 - 34.84)	29.59 ± 1.11 (24.93 - 35.75)	0.48 ± 0.65 (-4.44 - 5.64)	-0.82,1.79	0.461	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	3.07 ± 0.13 (2.39 - 3.44)	2.94 ± 0.13 (2.39 - 3.36)	0.13 ± 0.17 (-0.66 - 0.68)	-0.24,0.49	0.474	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.42 ± 0.046 (1.24 - 1.65)	1.42 ± 0.046 (1.16 - 1.61)	0.0015 ± 0.050 (-0.30 - 0.45)	-0.099,0.10	0.976	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	6.22 ± 0.23 (5.28 - 6.99)	6.26 ± 0.23 (5.37 - 6.80)	-0.036 ± 0.18 (-0.72 - 1.10)	-0.41,0.34	0.838	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	6.77 ± 0.42 (5.55 - 8.62)	6.63 ± 0.42 (2.72 - 9.02)	0.14 ± 0.36 (-2.35 - 3.83)	-0.64,0.91	0.714	(2.71 - 13.94) [0,20.49]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 17. Comparison of the Antinutrients and Secondary Metabolites Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Maize for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Antinutrient						
Phytic Acid (% DW)	0.75 ± 0.050 (0.53 - 0.87)	0.73 ± 0.050 (0.56 - 0.88)	0.016 ± 0.027 (-0.15 - 0.18)	-0.037,0.069	0.537	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	2131.38 ± 108.09 (1790.25 - 2525.31)	2148.05 ± 108.09 (1878.66 - 2669.85)	-16.67 ± 50.08 (-330.17 - 264.79)	-116.98,83.65	0.740	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	194.25 ± 7.12 (166.11 - 253.04)	183.96 ± 7.12 (167.76 - 210.13)	10.28 ± 7.08 (-24.37 - 70.84)	-4.73,25.30	0.165	(99.30 - 285.75) [0,378.57]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 18. Summary of Difference ($p<0.05$) for the Comparison of Analyte Levels for MON 89034 vs. the Conventional Control and Commercial References

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (Test minus Control) % of LH198 x LH172	Mean Difference (Test minus Control) Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Statistical Differences Observed in Combined Site Analyses						
Mineral						
Forage Phosphorus (% DW)	0.25	0.21	19.24	0.010	(0.22 - 0.32)	[0.071,0.32]
Fatty Acid						
Grain 18:0 Stearic (% Total FA)	1.89	1.82	3.97	0.002	(1.79 - 2.03)	[0.86,2.98]
Grain 20:0 Arachidic (% Total FA)	0.39	0.38	3.43	<0.001	(0.36 - 0.42)	[0.23,0.54]
Statistical Differences in More Than One Site						
Proximate						
Site IA Grain Carbohydrates (% DW)	83.38	84.52	-1.34	0.008	(83.29 - 83.55)	[81.08,88.80]
Site OH Grain Carbohydrates (% DW)	84.26	83.80	0.55	0.009	(83.99 - 84.59)	[81.08,88.80]
Mineral						
Site IL-1 Grain Copper (mg/kg DW)	1.76	1.36	29.35	0.023	(1.51 - 2.21)	[0,4.20]
Site NE Grain Copper (mg/kg DW)	2.15	1.67	28.66	0.023	(1.92 - 2.38)	[0,4.20]
Site IL-1 Grain Iron (mg/kg DW)	20.86	19.48	7.11	0.048	(19.23 - 21.79)	[8.88,34.51]
Site OH Grain Iron (mg/kg DW)	21.37	25.74	-17.00	0.006	(20.59 - 21.76)	[8.88,34.51]
Fatty Acid						
Site IL-1 Grain 18:0 Stearic (% Total FA)	1.96	1.82	7.94	<0.001	(1.89 - 2.02)	[0.86,2.98]
Site IL-2 Grain 18:0 Stearic (% Total FA)	1.98	1.82	9.05	<0.001	(1.93 - 2.03)	[0.86,2.98]
Site IL-1 Grain 20:0 Arachidic (% Total FA)	0.41	0.39	5.23	0.007	(0.40 - 0.42)	[0.23,0.54]

Table 18 (cont.). Summary of Difference ($p<0.05$) for the Comparison of Maize Component Levels for MON 89034 vs. the Conventional Control and Commercial Reference Substances

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (Test minus Control) % of LH198 x LH172	Mean Difference (Test minus Control) Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Fatty Acid (cont)						
Site IL-2 Grain 20:0 Arachidic (% Total FA)	0.39	0.37	6.83	0.021	(0.38 - 0.40)	[0.23,0.54]
Site OH Grain 20:0 Arachidic (% Total FA)	0.38	0.37	3.12	0.035	(0.38 - 0.39)	[0.23,0.54]
Statistical Differences Observed in One Site Only						
Amino Acid						
Site IA Grain Alanine (% DW)	0.88	0.81	7.83	0.030	(0.87 - 0.88)	[0.48,1.08]
Site IA Grain Arginine (% DW)	0.51	0.46	10.83	0.005	(0.50 - 0.52)	[0.33,0.56]
Site IA Grain Aspartic acid (% DW)	0.77	0.71	8.66	0.003	(0.77 - 0.78)	[0.43,0.90]
Site IA Grain Cystine (% DW)	0.25	0.23	7.54	0.014	(0.24 - 0.26)	[0.18,0.27]
Site IA Grain Glutamic acid (% DW)	2.27	2.09	8.66	0.011	(2.26 - 2.28)	[1.25,2.75]
Site IA Grain Glycine (% DW)	0.41	0.38	6.94	0.020	(0.40 - 0.41)	[0.28,0.46]
Site IA Grain Histidine (% DW)	0.34	0.32	7.16	0.022	(0.34 - 0.34)	[0.22,0.38]
Site IA Grain Leucine (% DW)	1.49	1.37	8.96	0.032	(1.48 - 1.51)	[0.77,1.92]
Site IA Grain Lysine (% DW)	0.35	0.32	6.66	0.028	(0.33 - 0.36)	[0.20,0.40]
Site IA Grain Methionine (% DW)	0.25	0.23	11.20	0.003	(0.25 - 0.27)	[0.14,0.25]
Site IA Grain Phenylalanine (% DW)	0.58	0.53	9.45	0.028	(0.57 - 0.59)	[0.32,0.73]

Table 18 (cont.). Summary of Difference (p<0.05) for the Comparison of Maize Component Levels for MON 89034 vs. the Conventional Control and Commercial Reference Substances

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (Test minus Control) % of LH198 x LH172	Mean Difference (Test minus Control) Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Amino Acid (cont)						
Site IA Grain Proline (% DW)	1.05	0.98	7.29	0.028	(1.04 – 1.05)	[0.68, 1.21]
Site IA Grain Serine (% DW)	0.60	0.56	8.28	0.004	(0.60 - 0.61)	[0.34,0.71]
Site IA Grain Threonine (% DW)	0.37	0.34	8.45	0.004	(0.37 - 0.37)	[0.24,0.41]
Site IA Grain Tyrosine (% DW)	0.43	0.36	17.50	0.006	(0.42 - 0.43)	[0.17,0.52]
Proximate						
Site IA Grain Protein (% DW)	11.89	10.85	9.59	0.005	(11.73 – 11.98)	[7.54,13.13]
Site IL-1 Forage Moisture (% FW)	69.03	66.53	3.76	0.031	(68.50 – 69.40)	[57.62,86.45]
Site NE Forage Ash (% DW)	3.20	4.39	-27.12	0.021	(2.93 – 3.38)	[1.93,6.31]
Site NE Forage Carbohydrates (% DW)	88.16	84.98	3.74	0.004	(86.86 – 88.84)	[83.05,90.74]
Fibre						
Site NE Grain Neutral Detergent Fibre (% DW)	10.52	9.05	16.27	0.028	(10.43 – 10.69)	[5.93,13.63]
Site OH Forage Acid Detergent Fibre (% DW)	31.31	23.58	32.78	0.012	(26.92 – 46.82)	[25.94,55.67]
Site OH Forage Neutral Detergent Fibre (% DW)	43.21	37.87	14.11	0.027	(40.07 – 46.82)	[25.94,55.67]

Table 18 (cont). Summary of Differences (p<0.05) for the Comparison of Maize Component Levels for MON 89034 vs. the Conventional Control and Commercial Reference Substances

Analytical Component (Units)[1]	Test Mean	Comparator Mean	Mean Difference (Test minus Comparator) % of Comparator	Mean Difference (Test minus Comparator) Signif. (p-Value)	Test (Range)	Commercial Tolerance Int.[2]
Fatty Acid						
Site IA Grain 18:3 Linolenic (% Total FA)	1.21	1.34	-9.40	0.009	(1.20 - 1.23)	[0.63,1.77]
Site IL-1 Grain 16:1 Palmitoleic (% Total FA)	0.13	0.14	-6.87	0.012	(0.12 - 0.13)	[0,0.28]
Site IL-2 Grain 18:1 Oleic (% Total FA)	24.75	23.82	3.93	0.003	(24.14 - 25.25)	[7.51,46.46]
Site IL-2 Grain 18:2 Linoleic (% Total FA)	61.87	63.17	-2.07	0.001	(61.19 - 62.42)	[39.41,76.74]
Site NE Grain 20:1 Eicosenoic (% Total FA)	0.28	0.29	-1.50	0.030	(0.28 - 0.28)	[0.15,0.39]
Mineral						
Site IA Grain Calcium (% DW)	0.0064	0.0058	10.96	0.012	(0.0062 - 0.0066)	[0.0016,0.0059]
Site IA Grain Manganese (mg/kg DW)	8.34	6.99	19.32	0.017	(7.62 - 9.32)	[3.17,9.99]
Site IA Forage Calcium (% DW)	0.24	0.26	-8.77	0.033	(0.24 - 0.24)	[0.016,0.38]
Site NE Forage Phosphorus (%DW)	0.25	0.17	46.95	0.036	(0.23 – 0.28)	[0.071,0.32]
Vitamin						
Site IL-2 Grain Folic Acid (mg/kg DW)	0.37	0.32	13.81	<0.001	(0.35 – 0.38)	[0.012,0.69]
Secondary Metabolite						
Site OH Grain p-Coumaric Acid (µg/g DW)	218.38	185.63	17.64	0.032	(187.79 – 253.04)	[0, 378.57]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; Combined Site = analyses of the combined data from each of the five replicated field trials.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 19. Literature and ILSI Database Ranges of Components of Maize Forage and Grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
Forage		
Proximates (% dw)		
Ash	2.43-9.64[a]; 2-6.6[b]	1.527 – 9.638
Carbohydrates	83.2-91.6[b]; 76.5-87.3[a]	76.4 – 92.1
Fat, total	0.35-3.62[b]; 1.42-4.57[a]	0.296 – 4.570
Moisture (% fw)	56.5-80.4[a];55.3-75.3[b]	49.1 – 81.3
Protein	4.98-11.56[a]	3.14 – 11.57
Fibre (% dw)		
Acid detergent fibre (ADF)	18.3-41.0[b]; 17.5-38.3[a]	16.13 – 47.39
Neutral detergent fibre (NDF)	26.4-54.5[b]; 27.9-54.8[a]	20.29 – 63.71
Minerals (% dw)		
Calcium	0.0969-0.3184[b]	0.0714 – 0.5768
Phosphorous	0.1367-0.2914[b]	0.0936 – 0.3704
Grain		
Proximates (% dw)		
Ash	1.1-3.9[d]; 0.89-6.28[b]	0.616 – 6.282
Carbohydrates	77.4-87.2[b]; 82.2-88.1[a]	77.4 – 89.5
Fat, total	3.1-5.7[d]; 2.48-4.81[b]	1.742 – 5.823
Moisture (% fw)	7-23[d]; 8.18-26.2[b]	6.1 – 40.5
Protein	6-12[d]; 9.7-16.1[c]	6.15 – 17.26
Fibre (% dw)		
Acid detergent fibre (ADF)	3.3-4.3[d]; 2.46-11.34[a,b]	1.82 – 11.34
Neutral detergent fibre (NDF)	8.3-11.9[d]; 7.58-15.91[b]	5.59 – 22.64
Total dietary fibre (TDF)	10.99-11.41[h]	8.82 – 35.31
Minerals		
Calcium (% dw)	0.01-0.1[d]	0.00127 – 0.02084
Copper (mg/kg dw)	0.9-10[d]	0.73 – 18.50
Iron (mg/kg dw)	1-100[d]	10.42 – 49.07
Magnesium (% dw)	0.09-1[d]	0.0594 – 0.194
Manganese (mg/kg dw)	0.7-54[d]	1.69 – 14.30
Phosphorous (% dw)	0.26-0.75[d]	0.147 – 0.533
Potassium (% dw)	0.32-0.72[d]	0.181 – 0.603
Zinc (mg/kg dw)	12-30[d]	6.5 – 37.2

Table 19 (cont). Literature and ILSI Database Ranges of Components of Maize Forage and Grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
Grain		
Amino Acids (% dw)		
Alanine	N/A	0.439 – 1.393
Arginine	N/A	**0.119 – 0.639**
Aspartic acid	N/A	0.335 – 1.208
Cystine	N/A	0.125 – 0.514
Glutamic acid	N/A	0.965 – 3.536
Glycine	N/A	0.184 – 0.539
Histidine	N/A	0.137 – 0.434
Isoleucine	N/A	0.179 – 0.692
Leucine	N/A	0.642 – 2.492
Lysine	N/A	0.172 – 0.668
Methionine	N/A	0.124 – 0.468
Phenylalanine	N/A	0.244 – 0.930
Proline	N/A	0.462 – 1.632
Serine	N/A	0.235 – 0.769
Threonine	N/A	0.224 – 0.666
Tryptophan	N/A	0.0271 – 0.215
Tyrosine	N/A	0.103 – 0.642
Valine	N/A	0.266 – 0.855
Fatty Acids	(% total fat)	(% total fatty acid)
16:0 Palmitic	7-19[e]	7.94 – 20.71
16:1 Palmitoleic	1[e]	0.095 – 0.447
18:0 Stearic	1-3[e]	1.02 – 3.40
18:1 Oleic	20-46[e]	17.4 – 40.2
18:2 Linoleic	35-70[e]	36.2 – 66.5
18:3 Linolenic	0.8-2[e]	0.57 – 2.25
20:0 Arachidic	0.1-2[e]	0.279 – 0.965
20:1 Eicosenoic	N/A	0.170 – 1.917
22:0 Behenic	N/A	0.110 – 0.349
Vitamins (mg/kg dw)		
Folic acid	0.3[d]	0.147 – 1.464
Niacin	9.3-70[d]	10.37 – 46.94
Vitamin B_1	3-8.6[e]	1.26 – 40.00
Vitamin B_2	0.25-5.6[e]	0.50 – 2.36
Vitamin B_6	5.3[d]; 9.6[e]	3.68 – 11.32
Vitamin E	3-12.1[e]; 17-47[d]	1.5 – 68.7

Table 19 (cont). Literature and ILSI Ranges of Components of Maize Forage and Grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
Grain		
Antinutrients (% dw)		
Phytic acid	0.48-1.12[a]	0.111 – 1.570
Raffinose	0.08-0.30[e]	0.020 – 0.320
Secondary Metabolites (µg/g dw)		
Ferulic acid	113-1194[f]; 3000[g]	291.9 – 3885.8
p-Coumaric acid	22-75[f]	53.4 – 576.2

[1]fw=fresh weight; dw=dry weight; Niacin =Vitamin B_3; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine ; N/A = not available as percent dry wt.
[2]Literature range references: [a]Ridley et al., 2002. [b]Sidhu et al., 2000. [c]Jugenheimer, 1976. [d]Watson, 1987. [e]Watson, 1982. [f]Classen et al., 1990. [g]Dowd and Vega, 1996. [h]Choi et al., 1999.
[3]ILSI range is from ILSI Crop Composition Database, 2006.

Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw

2.7 Nutritional Impact

There is no significant change in nutritional aspects of MON 89034 maize from the current commercial varieties of maize. Therefore there is no need to provide an indication of the likely dietary intake of MON 89034 according to the *Format for applying to amend the Australia New Zealand Food Standards Code – Food Produced using Gene Technology* (June 2005).

> *"If there have been any significant nutritional changes to the food, provide data on the human nutritional impact, including dietary patterns."*

2.8 Other Technical Information

a) Detection

MON 89034 produced the proteins Cry2Ab2 and Cry1A.105. The presence of the Cry2Ab2 protein in leaf tissue of MON 89034 plants can be detected through ELISA, or by the GeneCheck® assay. Cry1A.105 protein can be detected through ELISA. The materials and methods for the ELISA analysis are given in Hartmann et al., 2006 (Monsanto Study Report MSL-20285).

b) Projected Market Penetration

MON 89034 is a second-generation insect protection maize, developed to provide enhanced benefits for the control of lepidopteran pests. Compared to MON 810, MON 89034 will even better serve maize growers' need for controlling a wider spectrum of lepidopteran pests.

Maize is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa*) and wheat (*Titicum* sp.) in total global production. In 2004, maize was planted globally on 146.7 million hectares with a total production of 723.9 million metric tonnes (MMT) (FAOSTAT, 2006). The top three production countries in 2004 were: USA (299.9 MMT), China (130.4 MMT) and Brazil (41.8 MMT).

In industrialised countries maize has two major uses: (1) as animal feed in the form of grain, forage or silage; and 2) as a raw material for wet- and dry-milled processed products such as high fructose corn syrup, oil, starch, glucose, and dextrose. These processed products are used as ingredients in many industrial applications and in human food products. In developing countries, maize is used in a variety of ways. In Latin American countries such as Mexico, one of the main uses of maize is for food. In Africa, maize is consumed as a food in the sub-Saharan region, and in Asia it is generally used to feed animals (Morris, 1998).

There are no current plans to produce MON 89034 commercially in Australia or New Zealand. However, maize grain and products containing processed maize may potentially be imported into Australia and New Zealand from countries where MON 89034 is intended for commercialisation.

PART 3 REGULATORY / LEGISLATIVE IMPLICATIONS

3.1 Other Approvals

a) Overseas Regulatory Status

Monsanto has submitted a food and feed safety and nutritional assessment summary for MON 89034 to the United States Food and Drug Administration (FDA) in late 2006 and has also requested a Determination of Nonregulated Status for MON 89034 from the United States Department of Agriculture-Animal and Plant Health and Inspection Service and a request for tolerance exemption from the Environmental Protection Agency (EPA).

Regulatory submissions have been or will be made to countries that import significant maize or maize products, and have functional regulatory review processes in place. These include submissions to a number of additional governmental regulatory agencies including, but not limited to, Ministry of Agriculture (MOA) of China, Ministry of Health, Labor and Welfare (MHLW) and Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan, Canadian Food Inspection Agency (CFIA) and Health Canada, the Korean Food and Drug Administration (KFDA) and the Rural Development Administration (RDA), the Philippines Department of Agriculture and Taiwan's Department of Health (DOH).

b) Regulatory Rejection or Withdrawal

No application has been rejected or withdrawn by any regulatory body.

3.2 Regulatory Impact Statement

As described previously, maize is one of the most agriculturally important crops in the world. This application – if approved – will ensure any food imports from countries of MON 89034 maize production comply with the *Australian New Zealand Food Standards Code*. This will ensure that there is no potential for trade disruption on regulatory grounds.

PART 4 STATUTORY DECLARATION – AUSTRALIA

I, Alok Kumar, declare that the information provided in this application fully sets out the matters required and that the same are true to the best of my knowledge and belief, and that no information has been withheld that might prejudice this application.

Signature: Alok Kumar

Declared before me .....

This 13th day of Dec 2006.

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