Dow AgroSciences

Application to Amend the Food Standards Code - Food Produced Using Gene Technology

_B.t._ Cry1F Insect-resistant, Glufosinate-tolerant Maize Line 1507

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Part 1 - Information about the Applicant

1.1 The Applicant

This application is submitted by;

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The Managing Director of Dow AgroSciences Australia Pty. Ltd is;

Dow AgroSciences Australia Pty Ltd. is a wholly owned subsidiary of The Dow Chemical Company. Dow AgroSciences market science based products and systems which are used to protect food and fibre crops and urban and industrial buildings. Dow AgroSciences is a global company with headquarters in Indianapolis, Indiana USA. More than 3,500 staff are employed world-wide with a strong research program in conventional agricultural chemicals and biotechnology. Global Sales of Dow AgroSciences products is some US$3,000 million annually. The global web-site is found at www.dowagrosciences.com.

Dow AgroSciences' traditional crop protection products for the Australian and New Zealand markets are made in a plant in New Plymouth, New Zealand. This plant has ISO9002 accreditation and maintains quality standards and environmental procedures which exceed legal requirements. Dow AgroSciences was the first agricultural supplier in Australia to be ISO9002 accredited and one of the first to be NATA accredited to conduct GLP field trials.

Dow AgroSciences realised a major opportunity in biotechnology by purchasing Mycogen Corporation in 1998. Mycogen is one of the pioneers of agricultural biotechnology especially in the area of inserting genes of *Bacillus thuringiensis* into plants to create insect resistance. Their expertise has been a critical addition to the Dow AgroSciences overall biotech capabilities.

The Dow AgroSciences strategy to capturing value from biotechnology has been to invest in technology access rather than buying market share outright. Through acquisitions, alliances and licensing agreements, Dow AgroSciences has obtained the technology to deliver complex proteins in plants that will allow the production of pharmaceuticals, vaccines, feed and industrial ingredients from agricultural crops.

1.2 Nature of the Application

This application is submitted by Dow AgroSciences Australia Pty. Ltd on behalf of Mycogen Seeds, c/o Dow AgroSciences LLC (USA) (Mycogen Seeds) and Pioneer Hi-Bred International, Inc , a DuPont Company (Pioneer) as represented by Pioneer Overseas Corporation. Pioneer and Mycogen Seeds are the joint developers of the technology and producers of seed derived from *B.t.* Cry1F maize line 1507 (inbreds and hybrids).

The purpose of this application is to request that individual food safety approvals be granted to Pioneer and Mycogen Seeds for purposes of importing grain or grain products produced from *B.t.* Cry1F maize line 1507.

The addresses and contact names of both Pioneer and Mycogen Seeds are provided.

Pioneer Hi-Bred International, Inc, a DuPont Company
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Des Moines
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USA
Contact:

Mycogen Seeds
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Contact:

Part 2 - Data Required for Safety assessment

2.1 Background Details

Maize (*Zea mays* L.) is extensively cultivated and has a long history of safe use. Production has benefited from many improvements, particularly since the 1920's when maize varieties were developed by conventional breeding between progeny of two inbreds to give hybrid varieties which are superior to open-pollinated varieties in their agronomic characteristics, such as increased grain yield. Today, hybrid maize varieties are used in most developed countries for consistent agronomic performance and production (Canadian Food Inspection Agency 1994). In the case of maize line 1507maize line 1507 hybrids, the expression of CRY1F and PAT proteins will provide farmers additional improvements for insect pest and weed control.

Maize, together with rice and wheat, is one of the most important cereal crops in the world with total production of 591 million tonnes in 2000 (FAOSTAT Database 2001). Of this world wide production, some 253 million tonnes (43%) is produced in the USA. The majority of grain and forage derived from maize is used as animal feed. Maize grain is also processed into industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet-milling to produce starch and sweetener products. In addition to milling, the maize germ can be processed to obtain maize oil.

2.1a Description of the GM Organism

Describe the new GM organism from which the food being presented for safety assessment is derived. The description should include the nature and purpose of the genetic modification.

The organism is maize line 1507and progeny derived by conventional breeding. These maize lines express Cry1F protein for resistance to certain lepidopteran insect pests and pat protein for tolerance to glufosinate-ammonium herbicide.

Field research has shown that the Cry1F protein, as expressed in these maize lines, is very effective in controlling certain lepidopteran larva (such as European Corn Borer (ECB) *Ostrinia nubilalis* (Hubner), southwestern corn borer (SWCB) *Diatraea grandiosella*, black cutworm (BCW) *Agrotis ipsilon* and armyworms (*Spodoptera sp.*) that are common pests of corn in the USA where this maize will primarily be grown (Moellenbeck and Peters 1999).

An additional advantage accrued from the excellent control of insect pests using this technology is the reduction in moulds and associated mycotoxins which cost hundreds of millions of dollars in direct and indirect costs per year in the USA (Dowd 2000).

The original transformation event (designated TC1507) was produced by Pioneer using Dow AgroScience's plant optimized (po) modified *cry*1F gene derived from the *Bacillus thuringiensis* var. *aizawai* strain PS811. *Bacillus thuringiensis*, a common Gram-positive soil microorganism, produces an insecticidal protein that is very selective in toxicity to specific organisms. Decades of safety testing on *B.t.* protein support the lack of toxicity to humans and animals.

The *pat* gene, which encodes the enzyme phosphinothricin acetyltransferase, is also present in *B.t.* Cry1F maize line 1507. The *pat* gene is a modified version based on the native *pat* gene from *Streptomyces viridochromogenes*, a non-pathogenic microorganism. The inclusion of the *pat* gene enables plant selection of the transformed lines and provides tolerance to glufosinate-ammonium herbicides. The PAT protein does not confer pesticidal activity and there are no known adverse environmental or toxicological effects. *B.t.* Cry1F maize line 1507 is intended to be used by growers as a glufosinate tolerant line. Glufosinate has a history of safe use as a herbicide on maize in the United States.

2.1b GM Organism Identification

Provide the name, number or other identifier of each of the new lines or strains of GM organism from which the food is derived.

The organism will be identified as maize line 1507, which includes progeny derived by conventional breeding and expressing the traits for insect and herbicide tolerance. The family of traits will be referred to as Herculex™ *Insect Protection*.

2.1c Food Identity

Provide the name the food will be marketed under (if known)

There is no intention to market food items containing derived from maize line 1507 with specific brands or names.

2.1d Products containing the Food

List the type of products likely to include the food or food ingredient

The majority of grain and forage derived from maize is used for animal feeds. Less than 10% of maize grain is processed for human food products. Maize grain is also processed into industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet-milling to produce starch and sweetener products. In addition to milling, maize germ can be processed to obtain maize oil.

Domestic production of corn in Australia (*ca.* 340,000 t) and New Zealand is supplemented by import of a small amount of corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and corn chips. Other corn products such as maize starch are also imported. This is used by the food industry for the manufacture of dessert mixes and canned food.

2.2 History of Use

2.2a Donor Organisms

Describe all the donor organism(s) from which the genetic elements are derived including:

- *common and scientific names and taxonomic classification;*
- *information about any known pathogenicity, toxicity or allergenicity of relevance to the food;*
- *information about the history of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant)*

The DNA sequences present in the insert used for the genetic modification of maize line 1507 are derived from the following donors:

a. *ubi*ZM1(2) promoter, origin: *Zea mays* L. Other names: Maize, corn.

b. *cry*1F gene, origin: *Bacillus thuringiensis* sbsp. *aizawai.*

c. ORF25PolyA terminator sequence, origin: *Agrobacterium tumefaciens* strain pTi15955.

d. 35S promoter and terminator, origin: Cauliflower mosaic virus. Other names: Brassica virus 3, broccoli mosaic virus, cabbage mosaic virus, cabbage virus B, CaMV.

e. *pat* gene, origin: *Streptomyces viridochromogenes* strain Tü494.

Bacillus thuringiensis is a diverse group of Gram-positive, spore-forming bacteria that were first discovered in Japan on diseased silkworm in 1901 and has a history of decades of safe use as a pesticide (Schnepf *et al.* 1998; EPA 1996). Several varieties of *B. thuringiensis* have been used as microbial insecticides since 1938 (Merritt 1998). The subspecies *aizawai* is commercially used to control wax moth larvae and various caterpillars, especially the diamondback moth caterpillar (Cornell University 1996).

Streptomyces viridochromogenes, donor of the *pat* gene, is a common soil bacterium that produces the tripeptide L-phosphinothricyl-L-alanyl-alanine (L-PPT), which was developed as a non-selective herbicide by Hoechst Ag. There is a history of safe use of the *pat* gene in GM crops (OECD 1999). *Agrobacterium tumefaciens* is a Gram-negative, non-sporing, rod-shaped bacterium, closely related to *Rhizobium* which forms nitrogen-fixing nodules on clover and other leguminous plants. *Agrobacterium* contains a plasmid (the *Ti* plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally, *Agrobacterium* is a pathogen causing root deformation mainly with sugar beets, pomefruit and viniculture crops. However, the *Ti* plasmid has been altered, *i.e.* disarmed, to make it a useful vector for plant transformation by removing the sequences involved in pathogenicity and inserting sequences necessary for cloning and replication in *Escherichia coli* and/or for plant transformation.

The cauliflower mosaic virus, donor of the CaMV 35S promoter and terminator sequences, is a DNA caulimovirus with a host range restricted primarily to cruciferous plants (ICTV Database 1998). The DNA sequences originating from the cauliflower mosaic virus, the 35S promoter and terminator, have no pathological characteristics (USDA 1995).

For information on the potential toxicity or allergenicity of the donor organisms see sections 2.5e and 2.7.

2.2b Host Organism

Describe the host organism into which the genes were transferred and its history of safe use for food including:

- *any relevant phenotyptic information;*
- *how the organism is typically propagated for food use;*
- *what part of the organism is typically used as food;*
- *whether special processing is required to render food derived from the organism safe to eat;*
- *the significance to the diet in Australia and New Zealand of food derived from the host organism;*

Maize (*Zea mays* L.) is the only species usually included in the genus *Zea*, of the family Gramineae. It is a highly domesticated agricultural crop with well-characterised phenotypic and genetic traits. It reproduces sexually by wind-pollination and being a monoecious species has separate male staminate (tassels) and female pistillate (silk) flowers. This gives natural outcrossing between maize plants but it also enables the control of pollination in the production of hybrid seed. Typical of wind-pollinated plants, a large amount of redundant maize pollen is produced for each successful fertilisation of an ovule on the ear. Wind movements across the maize field cause pollen from the tassel to fall on the silks of the same or adjoining plants. Measuring about 0.1 mm in diameter, maize pollen is the largest of any pollen normally disseminated by wind from a comparably low level of elevation.

Repeated cycles of self-pollination leads to homogeneity of the genetic characteristics within a single maize plant (inbred). Controlled cross-pollination of inbred lines from chosen genetic pools combines desired genetic traits in a hybrid resulting in improved agronomic performance and yield increase. This inbred-hybrid concept and resulting yield response is the basis of the modern maize seed industry. Open pollination of hybrids in the field leads to the production of grain with properties from different lines and, if planted, would produce lower yields than those obtained with hybrids (Canadian Food Inspection Agency 1994).

Maize is extensively cultivated world-wide and has a long history of safe use. Production has benefited from many improvements, particularly since the 1920's when maize varieties were developed by conventional breeding between progeny of two inbreds to give hybrid varieties which are superior to open-pollinated varieties in their agronomic characteristics, such as increased grain yield. In present agricultural systems, hybrid maize varieties are used in most developed countries for consistent agronomic performance and production. In the case of maize line 1507 hybrids, the expression of CRY1F and PAT proteins will provide farmers additional improvements for insect pest and weed control.

Maize, together with rice and wheat, is one of the most important cereal crops in the world with total production of 591 million tonnes in 2000 (FAOSTAT Database 2001). Of this world wide production, some 253 million tonnes (43%) is produced in the USA. The majority of grain and forage derived from maize is used as animal feed. Maize grain is also processed into industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet-milling to produce

starch and sweetener products. In addition to milling, the maize germ can be processed to obtain maize oil and for numerous other minor uses (White and Pollak 1995). No special processing is required to make maize safe to feed or eat,

2.3 Nature of the Genetic Modification

2.3a Transformation Method

Describe the method used to transform the host organism.

The particle acceleration method (Klein *et al.* 1987) was used to introduce a purified linear DNA fragment (PHI8999A, 6235 bp) containing the *cry*1F and *pat* coding sequences and the necessary regulatory components (Figure 1) into maize cells resulting in maize event TC1507. The plant regenerated from these maize cells expressed the CRY1F protein and the PAT protein and is referred to as *B.t.* Cry1F maize line 1507, or simply as maize line 1507. Details of the transformation are described below (Narva *et al.* 1998).

Immature embryos isolated from Hi-II maize ears harvested soon after pollination were cultured on callus initiation medium for several days. On the day of transformation, microscopic tungsten particles were coated with the purified PHI8999A insert (Figure 1), containing the *cry*1F and *pat* genes, and they were accelerated into the cultured embryos, where the insert DNA was incorporated into the maize cell chromosome.

It is important to stress that only the purified insert PHI8999A consisting of a linear DNA fragment (6235 bp) containing the *cry*1F and *pat* coding sequences with necessary regulatory components for expression of CRY1F and PAT proteins in maize was inserted during the transformation of maize line 1507. No additional plasmid DNA was integrated in the transformation event. In particular, the *nptII* gene was not part of the 6235 bp linear DNA fragment (PHI8999A) that was purified and used in the transformation. The *nptII* gene is therefore not present in maize line 1507, as confirmed by the molecular analyses carried out with Southern blots (section 2.3d below).

After transformation, the embryos were transferred to callus initiation medium containing the herbicide glufosinate-ammonium as the selection agent for the expression of PAT protein. Individual embryos were kept physically separate during culture, and the majority of explants died on the selective medium.

Those embryos that survived and produced healthy, glufosinate-ammonium tolerant callus tissue were assigned unique identification codes representing putative events, and were continually transferred to fresh selection medium. Maize plants were regenerated from tissue derived from each unique event and transferred to the greenhouse. Leaf samples were taken for molecular analysis to verify the presence of the inserted genes by PCR and to confirm expression of the CRY1F protein by ELISA. Plants were then subjected to a whole plant bioassay using European corn borer insects. Positive plants were crossed with inbred lines to obtain seed from the initial transformed plants. A number of lines were evaluated in the field. Maize line 1507 (event TC1507) was selected for its good agronomic characteristics and excellent resistance to European corn borer and other lepidopteran insect pests.

2.3b Bacteria used for manipulation

Provide information about the bacteria used for all the laboratory manipulations prior to transformation of the host organism.

Not applicable

2.3c Gene Construct and Vectors

Describe the gene construct and the transformation vectors used, including;

- *the size, source and function of all the genetic components including marker genes, regulatory and other elements;*
- *a detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites.*

The insert, PHI8999A, consisted of a linear DNA fragment of 6235 bp containing the plant optimized and truncated *cry1*F gene and the plant optimized *pat* gene together with the regulatory sequences necessary for their expression in maize plants, as shown in Figure 1 and further described in Table 1. The insert was obtained from plasmid PHP8999 (9504 bp, Figure 2). Digestion of the plasmid DNA with the restriction enzyme *Pme*I provided the 6235 bp insert, which was subsequently purified by agarose gel electrophoresis. The remaining 3269 bp fragment containing the *nptII* gene was discarded and therefore not used in the transformation.

Figure 1: Restriction map of the 6235 bp insert PHI8999A used in the transformation of maize line 1507

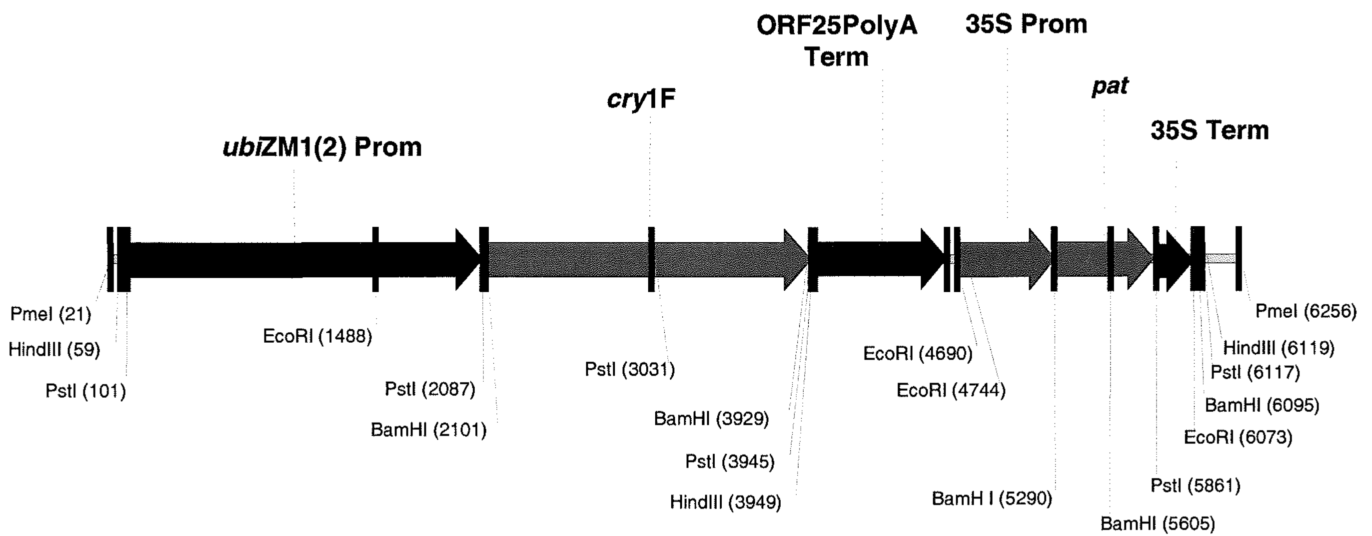

Figure 2: Plasmid map of PHP8999 used in the construction of insert PHI8999A

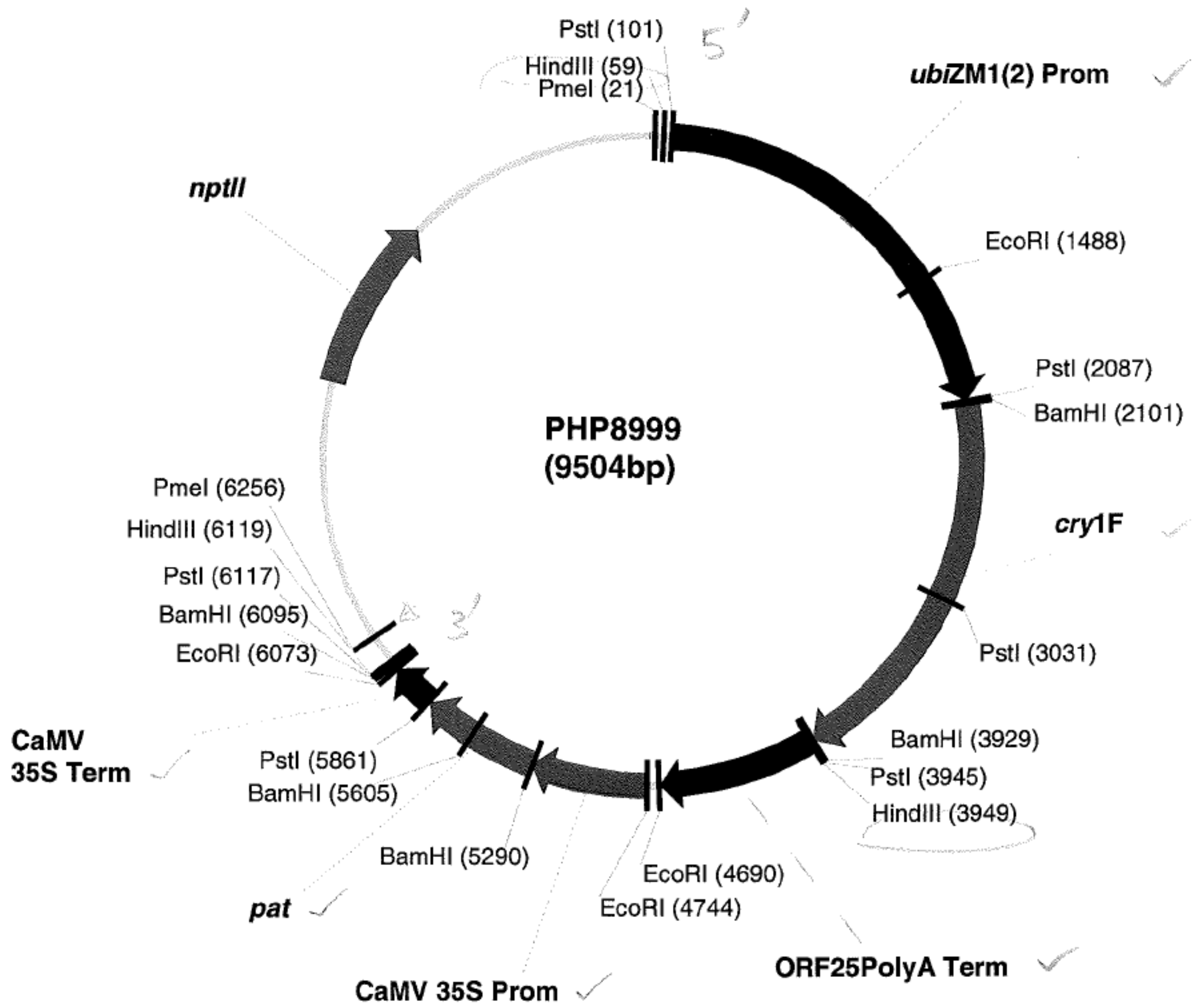

Table 1: Genetic elements in insert PHI8999A used to transform maize line 1507

Location on insert PHI8999A (bp to bp incl.)	Location on plasmid PHP8999 (bp to bp incl.)	Genetic element	Size (bp incl.)	Function
1 – 80	21 – 100	Polylinker region	80	Contains restriction sites required for cloning of the genetic elements
81 – 2066	101 – 2086	*ubi*ZM1(2)	1986	The ubiquitin promoter (plus 5' untranslated region) from *Zea mays* (Christensen *et al.* 1992)
2067 – 2089	2087 – 2109	Polylinker region	23	Contains restriction sites required for cloning of the genetic elements
2090 – 3907	2110 – 3927	*cry*1F	1818	A synthetic version of truncated *cry*1F from *Bacillus thuringiensis* sbsp. *aizawai* (plant optimized)
3908 – 3953	3928 – 3973	Polylinker region	46	Contains restriction sites required for cloning of the genetic elements
3954 – 4667	3974 – 4687	ORF25PolyA	714	A terminator from *Agrobacterium tumefaciens* pTi15955
4668 – 4723	4688 – 4743	Polylinker region	56	Contains restriction sites required for cloning of the genetic elements
4724 – 5277	4744 – 5297	CaMV 35S promoter	554	35S promoter from Cauliflower Mosaic Virus (Odell *et al.* 1985)
5278 – 5829	5298 – 5849	*pat*	552	The synthetic glufosinate-ammonium tolerance gene (plant optimized), based on a phosphinothricin acetyltransferase gene sequence from *Streptomyces viridochromogenes* (Wohlleben *et al.* 1988; Eckes *et al.* 1989)
5830 – 5846	5850 – 5866	Polylinker region	17	Contains restriction sites required for cloning of the genetic elements
5847 – 6050	5867 – 6070	CaMV 35S terminator	204	35S terminator from Cauliflower Mosaic Virus (Pietrzak.M. *et al.* 1986)
6051 – 6235	6071 – 6255	Polylinker region	185	Contains restriction sites required for cloning of the genetic elements

No vector was used for the transformation of maize line 1507. As mentioned above, a linear DNA fragment containing the *cry1*F and *pat* coding sequences together with the necessary regulatory components only was used for transformation by particle acceleration. No carrier DNA sequences were used for transformation of the insert.

A complete description of the genetic elements contained in the insert is presented in Table 1. The sequence of the truncated *cry1*F gene has been optimized for plant codon usage. The sequence of the *pat* gene has also been optimized for expression in plants (OECD 1999).

The amino acid sequence of the core CRY1F protein expressed in maize line 1507 is shown in Figure 3. With the exception of a single amino acid substitution, leucine at position 604 ($F_{604}L$ substitution in Figure 3), it is identical to amino acids 1-605 of the native CRY1F protein which is 1174 amino acids long. This change in the coding sequence was made to introduce a *Xho*I restriction site for fusion of sequences encoding the C-terminal domain of the protein that forms the full length protein. The choice to use the $F_{604}L$ substitution was based on the occurrence of leucine in the homologous position of other CRY1 proteins (Evans 1998; Narva *et al.* 1998).

The amino acid sequence of the microbially-derived CRY1F protein (MR872) used in toxicological studies, is also included for comparison in Figure 3. The MR872 designation is given to the chimeric CRY1F/CRY1A(b) protein produced in *Pseudomonas fluorescens* for toxicological studies. The sequence coding for the CRY1A(b) C-terminal domain was fused with the sequence coding for the CRY1F core toxin to enable high expression in *Pseudomonas fluorescens*. The microbially-derived CRY1F/CRY1A(b) protein was treated with trypsin to obtain the CRY1F core protein for use in toxicity studies having shown that its biochemical characteristics and biological activity are equivalent to the maize expressed core CRY1F protein (Evans 1998). The core CRY1F protein expressed in maize represents amino acids 1 to 605. The positions of putative protease cleavage sites at the start (about 28 or 31) and at the end (about residue 612 or 615) of the active core toxin are marked with a ↓ (Figure 3). The test sample used in the toxicity studies is therefore comparable to the core CRY1F protein expressed in maize line 1507 (section 2.5e).

Figure 3: Comparison of the amino acid sequences of microbially-derived CRY1F protein (MR872); CRY1F protein from maize line 1507 (CRY1Fsyn); and, CRY1F protein from *Bacillus thuringiensis* sbsp. *aizawai*. The positions of putative protease cleavage sites at the start (about residue 28 or 31) and end (about residue 612 or 615) of the active core toxin are marked with an ↓.

MR872 = Amino acid sequence of microbially-derived CRY1F protein used in toxicological studies.
CRY1Fsyn = Amino acid sequence of CRY1F protein from maize line 1507.
CRY1F = Amino acid sequence of CRY1F protein from *Bacillus thuringiensis* sbsp. *aizawai*

```
           1                                                 50
MR872      MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
CRY1Fsyn   MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
CRY1F      MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
Consensus  MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP

           51                                               100
MR872      GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
CRY1Fsyn   GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
CRY1F      GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
Consensus  GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG

           101                                              150
MR872      LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
CRY1Fsyn   LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
CRY1F      LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
Consensus  LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS

           151                                              200
MR872      FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
CRY1Fsyn   FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
CRY1F      FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
Consensus  FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR

           201                                              250
MR872      YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
CRY1Fsyn   YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
CRY1F      YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
Consensus  YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV

           251                                              300
MR872      RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
CRY1Fsyn   RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
CRY1F      RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
Consensus  RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM

           301                                              350
MR872      NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
CRY1Fsyn   NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
CRY1F      NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
Consensus  NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE

           351                                              400
MR872      DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
CRY1Fsyn   DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
CRY1F      DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
Consensus  DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI

           401                                              450
MR872      DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
CRY1Fsyn   DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
CRY1F      DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
Consensus  DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH

           451                                              500
MR872      RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
CRY1Fsyn   RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
CRY1F      RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
Consensus  RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP

           501                                              550
MR872      FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
CRY1Fsyn   FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
CRY1F      FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
Consensus  FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
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```
           551                                                    600
     MR872 GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
  CRY1Fsyn GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
     CRY1F GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
 Consensus GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV

           601                                                    650
     MR872 TATFEAEYDL ERAQKAVNAL FTSINQIGIK TDVTDYHIDR VSNLVECLSD
  CRY1Fsyn TATLE*.... .......... .......... .......... ..........
     CRY1F TATFEAEYDL ERAQKAVNAL FTSINQIGIK TDVTDYHIDQ VSNLVDCLSD
 Consensus TAT-E----- ---------- ---------- ---------- ----------

           651                                                    700
     MR872 EFCLDEKKEL SEKVKHAKRL SDERNLLQDP NFRGINRQLD RGWRGSTDIT
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F EFCLDEKREL SEKVKHAKRL SDERNLLQDP NFKGINRQLD RGWRGSTDIT
 Consensus ---------- ---------- ---------- ---------- ----------

           701                                                    750
     MR872 IQGGDDVFKE NYVTLLGTFD ECYLTYLYQK IDESKLKAYT RYQLRGYIED
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F IQRGDDVFKE NYVTLPGTFD ECYPTYLYQK IDESKLKPYT RYQLRGYIED
 Consensus ---------- ---------- ---------- ---------- ----------

           751                                                    800
     MR872 SQDLEIYLIR YNAKHETVNV PGTGSLWRLS APSPI..... ..........
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F SQDLEIYLIR YNAKHETVNV LGTGSLWPLS VQSPIRKCGE PNRCAPHLEW
 Consensus ---------- ---------- ---------- ---------- ----------

           801                                                    850
     MR872 .......... .GKCAHHSHH FSLDIDVGCT DLNEDLGVWV IFKIKTQDGH
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F NPDLDCSCRD GEKCAHHSHH FSLDIDVGCT DLNEDLDVWV IFKIKTQDGH
 Consensus ---------- ---------- ---------- ---------- ----------

           851                                                    900
     MR872 ARLGNLEFLE EKPLVGEALA RVKRAEKKWR DKREKLEWET NIVYKEAKES
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F ARLGNLEFLE EKPLVGEALA RVKRAEKKWR DKREKLELET NIVYKEAKES
 Consensus ---------- ---------- ---------- ---------- ----------

           901                                                    950
     MR872 VDALFVNSQY DRLQADTNIA MIHAADKRVH SIREAYLPEL SVIPGVNAAI
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F VDALFVNSQY DQLQADTNIA MIHAADKRVH RIREAYLPEL SVIPGVNVDI
 Consensus ---------- ---------- ---------- ---------- ----------

           951                                                   1000
     MR872 FEELEGRIFT AFSLYDARNV IKNGDFNNGL SCWNVKGHVD VEEQNNHRSV
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F FEELKGRIFT AFFLYDARNV IKNGDFNNGL SCWNVKGHVD VEEQNNHRSV
 Consensus ---------- ---------- ---------- ---------- ----------

           1001                                                  1050
     MR872 LVVPEWEAEV SQEVRVCPGR GYILRVTAYK EGYGEGCVTI HEIENNTDEL
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F LVVPEWEAEV SQEVRVCPGR GYILRVTAYK EGYGEGCVTI HEIENNTDEL
 Consensus ---------- ---------- ---------- ---------- ----------

           1051                                                  1100
     MR872 KFSNCVEEEV YPNNTVTCND YTATQEEYEG TYTSRNRGYD GAYESNSSVP
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F KFSNCVEEEV YPNNTVTCND YTANQEEYGG AYTSRNRGYD ETYGSNSSVP
 Consensus ---------- ---------- ---------- ---------- ----------

           1101                                                  1150
     MR872 ADYASAYEEK AYTDGRRDNP CESNRGYGDY TPLPAGYVTK ELEYFPETDK
  CRY1Fsyn .......... .......... .......... .......... ..........
     CRY1F ADYASVYEEK SYTDGRRDNP CESNRGYGDY TPLPAGYVTK ELEYFPETDK
 Consensus ---------- ---------- ---------- ---------- ----------

           1151                  1175
     MR872 VWIEIGETEG TFIVDSVELL LMEE*
  CRY1Fsyn .......... .......... .....
     CRY1F VWIEIGETEG TFIVDSVELL LMEE*
 Consensus ---------- ---------- -----
```

2.3d Molecular Characterisation of the Modification

Provide a full molecular characterisation of the genetic modification in the new organism, including:

- *identification of all transferred genetic material and whether it has undergone any rearrangements;*
- *a determination of the number of insertion sites, including the number of copies at each insertion site;*
- *full DNA sequence data of each insertion event; including junction regions with the host DNA;*
- *a map depicting the organisation of the inserted genetic material at each insertion site;*
- *the identification and characterisation of any unexpected open reading frames, including those that could result in fusion proteins or unexpected protein expression products.*

The insert is integrated into the maize plant genome as confirmed by molecular characterization of maize line 1507 by Southern blot analyses (described below) and based on a detailed study (Glatt 2000).

The Southern blot analyses demonstrate that maize line 1507 contains:

1. The synthetic version of plant optimized and truncated *cry*1F gene from *Bacillus thuringiensis* sbsp. *aizawai* with transcription directed by the ubiquitin promoter from *Zea mays* and with a termination sequence derived from ORF25PolyA from *Agrobacterium tumefaciens* pTi15995.

2. The synthetic glufosinate-ammonium tolerance gene (plant optimized), based on a phosphinothricin acetyltransferase gene sequence, *pat*, from *Streptomyces viridochromogenes* with transcription directed by CaMV 35S promoter and CaMV 35S terminator, from cauliflower mosaic virus.

The results of the detailed molecular characterization support the conclusion that maize line 1507 contains a full-length copy of the DNA insert used in the transformation (i.e., the 6235 bp fragment of insert PHI8999A containing the *cry*1F and *pat* genes) and an additional copy of the *cry*1F gene (Glatt 2000).

Leaf samples were obtained from plants of two different generations during the breeding of maize line 1507: designated as T1S1 generation and BC4 generation. The T1S1 generation seed consisted of the original transformed Hi-II maize line crossed to an elite inbred to give an F1 hybrid, and then selfed to give T1S1 seed. The BC4 generation seed consisted of the fourth backcross generation of the original transformed Hi-II maize line. Plants of both generations were grown in the glasshouse and leaf samples obtained for genomic DNA extraction and analysis.

Plasmid PHP8999 DNA, genomic DNA from Hi-II maize, and genomic DNA from maize line 1507 T1S1 and BC4 generations were digested with the restriction enzymes *Pme*I, *Hin*dIII, *Pst*I, *Bam*HI, *Eco*RI, and *Bam*HI combined with *Eco*RI. Genomic DNA lots within each maize line 1507 generation were pooled prior to digestion. Representative aliquots of each digest were separated on five individual agarose gels and transferred to nylon membranes. A description of the DNA probes used in this study is shown in Table 2.

Table 2: Summary of DNA probe sizes and locations relative to plasmid PHP8999 (Glatt 2000).

DNA probe	Location on insert PHI8999A (bp to bp)	Location on plasmid PHP8999 (bp to bp)	Size (bp)	Comments
ubi	100 – 1687	120 – 1707	1587	Hybridizes to the *ubi*ZM1(2) promoter for the *cry*1F gene
*cry*1F	2528 – 3507	2548 – 3527	979	Hybridizes to coding region for the *cry*1F gene
CaMV 35S	4771 – 5209	4791 – 5229	438	Hybridizes to the CaMV 35S promoter for the *pat* gene
pat	5517 – 5826	5537 – 5846	309	Hybridizes to coding region for the *pat* gene
nptII	Not present	7497 – 8033	536	Hybridizes to coding region for the *nptII* gene

The expected results for each digestion and hybridization are shown in Table 3, and the observed results for each digestion and hybridization are shown in Table 4. The expected fragment sizes in Table 3 assume that the DNA used to transform maize line 1507 was inserted into the genome as a single intact copy.

As expected, the *Pme*I restriction site is lost during transformation because the specific sequence required for *Pme*I digestion (GTTT/AAAC) is not likely to be present at the point of integration into the maize genome. Therefore, the expected and observed hybridization fragments are larger than 6235 bp (Tables 3 and 4).

Table 3: Summary of expected hybridizing fragments during Southern analyses of the DNA insert of maize line 1507 (Glatt 2000).

	Expected hybridizing fragment size in base pairs for each DNA probe				
Restriction enzyme	***ubi***	***cry1*F**	**CaMV 35S**	***pat***	***nptII***
*Pme*I	>6235	>6235	>6235	>6235	No fragments expected
*Hind*III	3890	3890	2170	2170	No fragments expected
*Pst*I	1986	914 944	1916	1916	No fragments expected
*Bam*HI	>2080	1828	1361	315 490	No fragments expected
*Eco*RI	>1467	3202	1329	1329	No fragments expected
*Bam*HI/*Eco*RI	>1467	1828	546	315 468	No fragments expected

Table 4: Summary of observed fragments during Southern analyses of the DNA insert of maize line 1507 (Glatt 2000).

	Observed hybridizing fragment size in base pairs for each DNA probe				
Restriction enzyme	***ubi***	***cry1*F**	**CaMV 35S**	***pat***	***nptII***
*Pme*I	~23000 [a,b]	~23000 [a,b]	~23000 [b]	~23000 [b]	No fragments observed
*Hind*III	~3890 [b] ~6500 [a] ~20000 [c]	~1000 [a] ~2000 [a] ~3890 [b] ~4000	~2170 [b]	~2170 [b]	No fragments observed
*Pst*I	~1986 [a,b] ~23000 [a]	~914 [b] ~944 [b] ~6500 ~23000 [a]	~1916 [b]	~1916 [b]	No fragments observed

*Bam*HI	~9000 [a] ~15000 [a] ~20000 [a]	~1828 [b] ~8000	~1361 [b]	~315 [b] ~490 [b]	No fragments observed
*Eco*RI	~1700 [a] ~3000 ~3500 ~4000 ~4100 [a] ~6500 [c] ~9400 ~23000	~3000 ~3202 [b] ~23000	~1329 [b]	~1329 [b]	No fragments observed
*Bam*HI/*Eco*RI	~1700 [a] ~3000 ~4000 [a] ~6500 [c] ~9000	~1828 [b] ~3000 ~5000 [a] ~8000	~546 [b]	~315 [b] ~468 [b]	No fragments observed

a: Similar fragment observed in negative control genomic DNA
b: DNA fragment predicted based on sequence of plasmid PHP8999
c: Detected in negative control genomic DNA only

Confirmation for presence of *cry*1F gene:

The digestions with *Hin*dIII, *Pst*I, and *Bam*HI were conducted for the purpose of characterizing the *cry*1F gene and its *ubi*ZM1(2) promoter in maize line 1507. The *Hin*dIII restriction enzyme cuts at the 5' end of the *ubi*ZM1(2) promoter and the 3' end of the *cry*1F coding sequence. The purpose of the *Hin*dIII digestion was to determine whether the full-length *cry*1F gene is present with its promoter intact. The *Pst*I digestion was intended to provide further information on whether the *ubi*ZM1(2) promoter was intact as this enzyme cuts essentially at both ends of this promoter. *Bam*HI digestion was intended to provide information on whether the *cry*1F coding sequence was intact as this enzyme cuts at both the 5' and 3' end of this coding sequence.

The expected results for the *cry*1F gene can be summarized as follows. *Hin*dIII digestion was expected to produce a 3890 bp fragment containing the *ubi*ZM1(2) promoter and *cry*1F gene. This expected fragment was observed after hybridization with probes specific for the *ubi*ZM1(2) promoter and *cry*1F (Figures 4 and 5). The *Pst*I digestion supports the conclusion that *ubi*ZM1(2) promoter is intact resulting in the expected 1986 bp fragment when genomic DNA was hybridized with the *ubi* probe (Figure 4). Finally, the expected 1828 bp fragment was present when genomic DNA was digested with *Bam*HI and hybridized with the *cry*1F probe, which indicated an intact *cry*1F coding sequence is present (Figure 5). Table 4 summarizes these results for the *cry*1F gene and its promoter.

The evidence for an additional copy of the *cry*1F sequence is based on results of the *Hin*dIII and *Pst*I digestions followed by hybridization with the *cry*1F and *ubi* probes as shown in Table 4. *Hin*dIII digestion and hybridization with the *cry*1F probe resulted in two bands: one of the expected 3890 bp size and a second, representing an additional copy, that is larger and estimated at ~4000 bp in size (Figure 5). Hybridization of the *Hin*dIII digest with the *ubi* probe resulted in one band of the expected 3890 bp size and failed to reveal the ~4000 bp fragment (Figure 4). This indicates that the promoter region is either absent in this additional copy or it is not intact. A small portion of the *ubi*ZM1(2) promoter cannot be detected by the *ubi* DNA probe used in this study because the *ubi* probe was prepared with a fragment of the *ubi*ZM1(2) promoter extending from 120 bp to 1707 bp (Table 2). Therefore, an approximately 300 bp region of the *ubi*ZM1(2) promoter that is 5' to the *cry*1F gene cannot be detected with this probe. None of the other digestions were designed to provide evidence for the presence or absence of the *ubi*ZM1(2) promoter on the additional *cry*1F gene. Interpretation of hybridization results with the *ubi* probe is made difficult by the fact that the *ubi*ZM1(2) promoter was isolated from maize and therefore is present in the non-GM control maize plants. This results in hybridizing bands that appear in the lanes containing DNA from the non-GM control and maize line 1507 (Figure 4 and Table 4). Nevertheless, the results of the *Hin*dIII digestion support the conclusion that the *ubi*ZM1(2) promoter on the additional copy of the *cry*1F coding sequence is either absent or not intact.

Confirmation for presence of *pat* gene:

The digestions with *Eco*RI, *Bam*HI, and the combination *Bam*HI/*Eco*RI, were conducted for the purpose of characterizing the *pat* gene and its CaMV promoter in maize line 1507. The *Eco*RI enzyme cuts at the 5' end of the CaMV 35S promoter and at the 3' end of the CaMV 35S terminator for the *pat* gene and was expected to result in a 1329 bp fragment if an intact copy of the *pat* gene and its CaMV 35S promoter and terminator was present in maize line 1507. The *Bam*HI enzyme cuts at the 5' end of the *pat* gene and within approximately 150 bp of the 3' end of this gene. An additional digestion with the combination *Bam*HI/*Eco*RI was conducted to determine whether a 546 bp fragment corresponding to the CaMV 35S promoter could be detected after hybridization with the CaMV 35S promoter DNA probe.

The results for the *pat* gene can be summarized as follows. The expected 1329 bp *Eco*RI fragment was observed after hybridization with the CaMV 35S and *pat* DNA probes (Figures 6 and 7). The presence of an intact CaMV 35S promoter was confirmed because the expected 546 bp fragment was observed with the combined *Bam*HI/*Eco*RI digestion (Figure 6). The presence of an intact *pat* gene was confirmed because the expected fragments were observed after *Bam*HI digestion followed by hybridization with the *pat* DNA probe (Figure 7). Finally, *Hin*dIII digestion was expected to produce 2170 bp fragment containing the CaMV 35S promoter, *pat* gene, and CaMV 35S terminator if the sequences were present as full-length copies. This expected fragment was observed after hybridization with the CaMV 35S and *pat* DNA probes (Figures 6 and 7). Table 4 summarizes these results for the *pat* gene and its promoter and terminator.

Confirmation for absence of *nptII* gene:

The *Pme*I DNA fragment used to transform maize line 1507 was obtained from plasmid PHP8999. The portion of the plasmid that was used for transformation did not contain the kanamycin resistance gene, *nptII*. To further verify that maize line 1507 does not contain the *nptII* gene, genomic DNA was hybridized with an *nptII* probe (Table 2). The Southern blot is shown in Figure 8. As expected, no bands hybridizing to the *nptII* DNA probe were detected. This confirms that the *nptII* gene for kanamycin resistance is not present in maize line 1507.

Conclusion:
The results of the detailed molecular characterization supports the conclusion that maize line 1507 contains a full-length copy of the DNA insert used in transformation (i.e., the 6235 bp fragment of insert PHI8999A containing the *cry*1F and *pat* genes) and an additional copy of the *cry*1F gene. As expected, the data support the conclusion that the *nptII* gene is not present in maize line 1507.

Sequence Information

It is anticipated that the final report detailing full sequence data, including DNA bordering the insert, will be avialable in October 2001.

Figure 4: Southern analysis of the *ubi*ZM1(2) promoter for the *cry1*F gene in the DNA insert of maize line 1507.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I and *Bam*H I/*Eco*R I. The lanes contain:

1: GS3 non-transgenic control (5µg)
2: pooled T1S1 DNA (5µg)
3: pooled BC4 DNA (5µg)
4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
M: molecular weight marker (*Hind* III digest of lambda DNA) (1µg). MW of lambda DNA fragments is given in kilobases (kb).

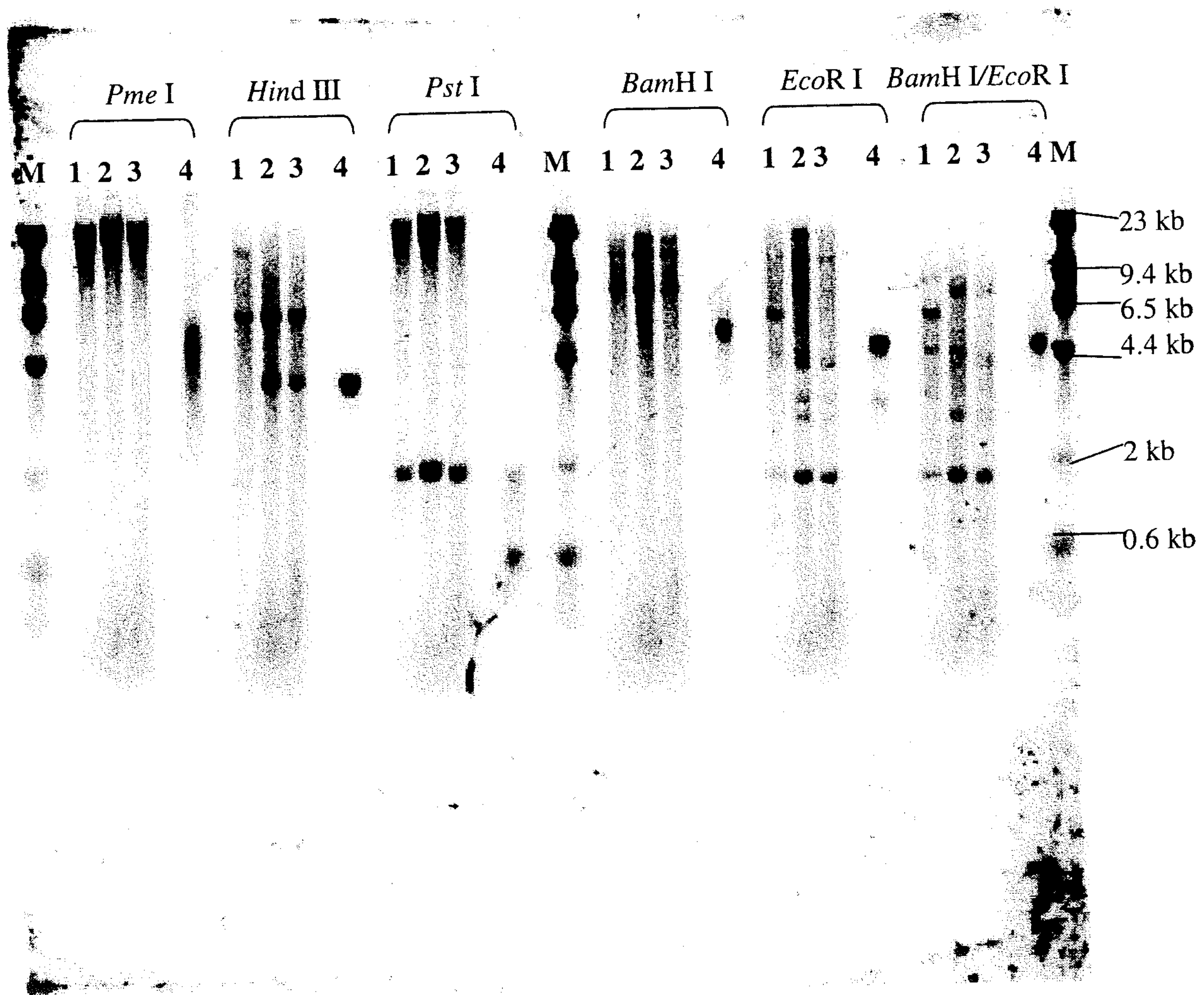


Figure 5: Southern analysis of the *cry1*F gene in the DNA insert of maize line 1507.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hin*d III, *Pst* I, *Bam*H I, *Eco*R I and *Bam*H I/*Eco*R I. The lanes contain:

1: GS3 non-transgenic control (5µg)
2: pooled T1S1 DNA (5µg)
3: pooled BC4 DNA (5µg)
4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
M: molecular weight marker (*Hin*d III digest of lambda DNA) (1µg). MW of lambda DNA fragments is given in kilobases (kb).

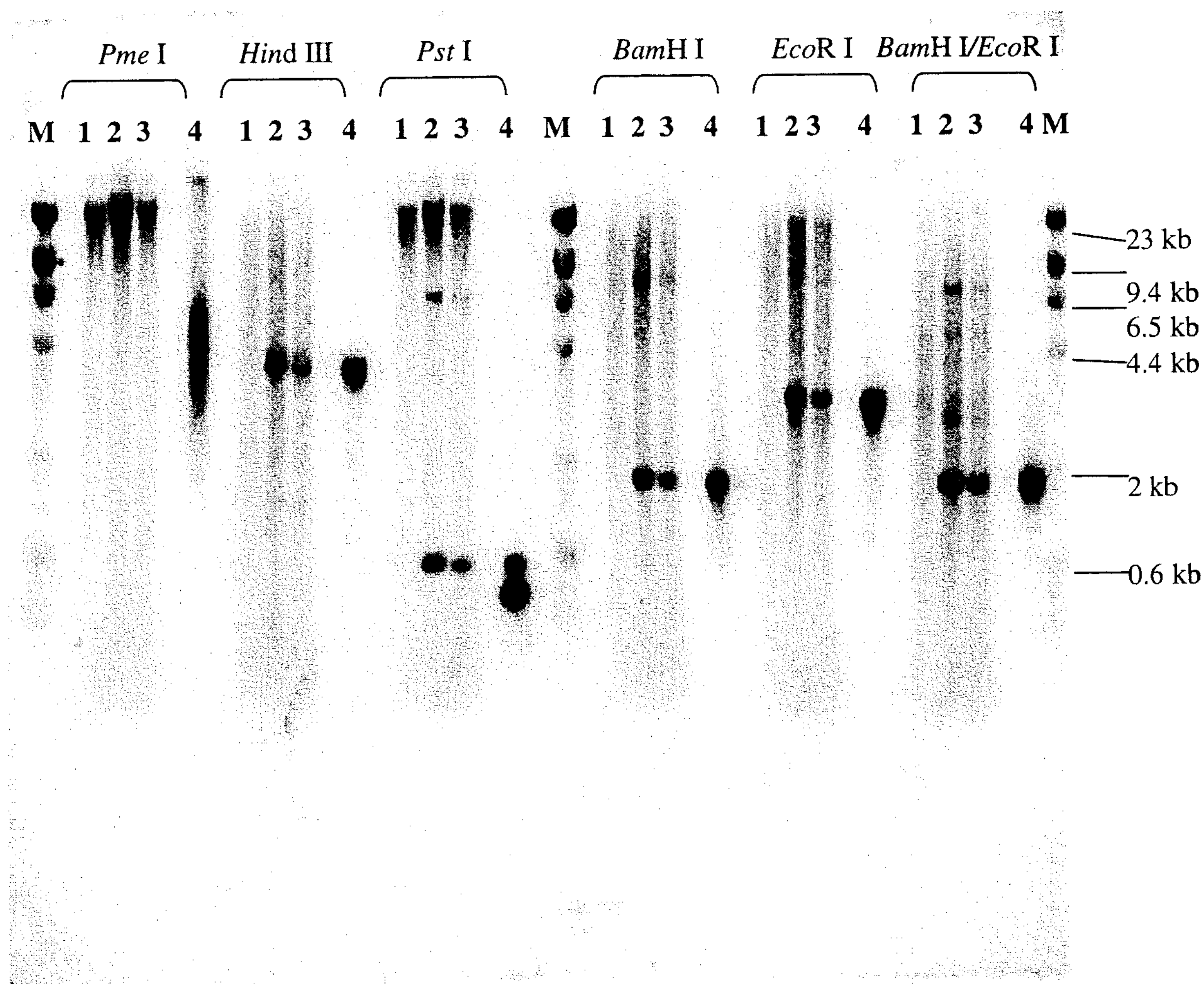


Figure 6: Southern analysis of the CaMV 35S promoter for the *pat* gene in the DNA insert of maize line 1507.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I and *Bam*H I/*Eco*R I. The lanes contain:

1: GS3 non-transgenic control (5µg)
2: pooled T1S1 DNA (5µg)
3: pooled BC4 DNA (5µg)
4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
M: molecular weight marker (*Hind* III digest of lambda DNA) (1µg). MW of lambda DNA fragments is given in kilobases (kb).

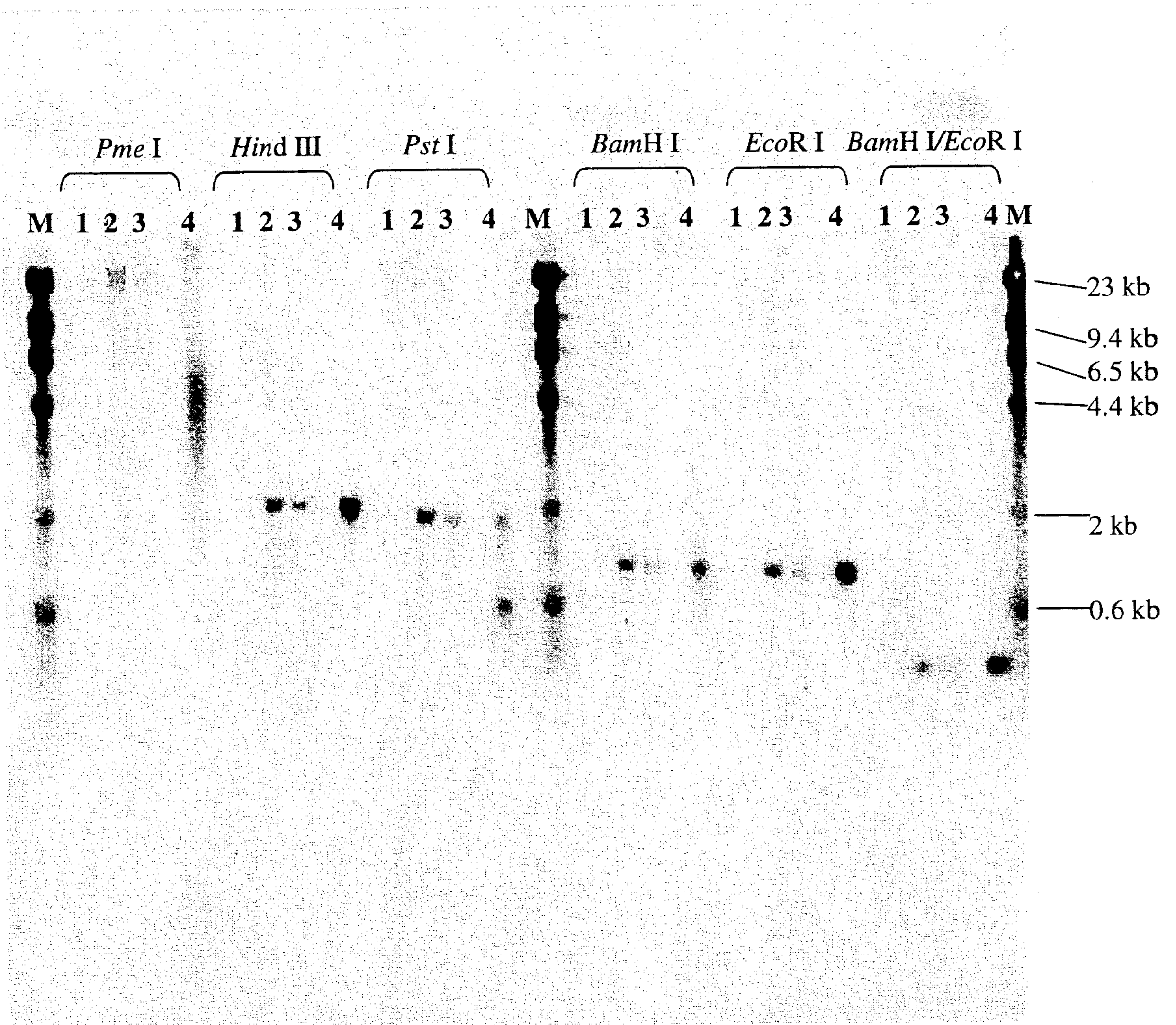


Figure 7: Southern analysis of the *pat* gene in the DNA insert of maize line 1507.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I and *Bam*H I/*Eco*R I. The lanes contain:

1: GS3 non-transgenic control (5µg)
2: pooled T1S1 DNA (5µg)
3: pooled BC4 DNA (5µg)
4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
M: molecular weight marker (*Hind* III digest of lambda DNA) (1µg). MW of lambda DNA fragments is given in kilobases (kb).

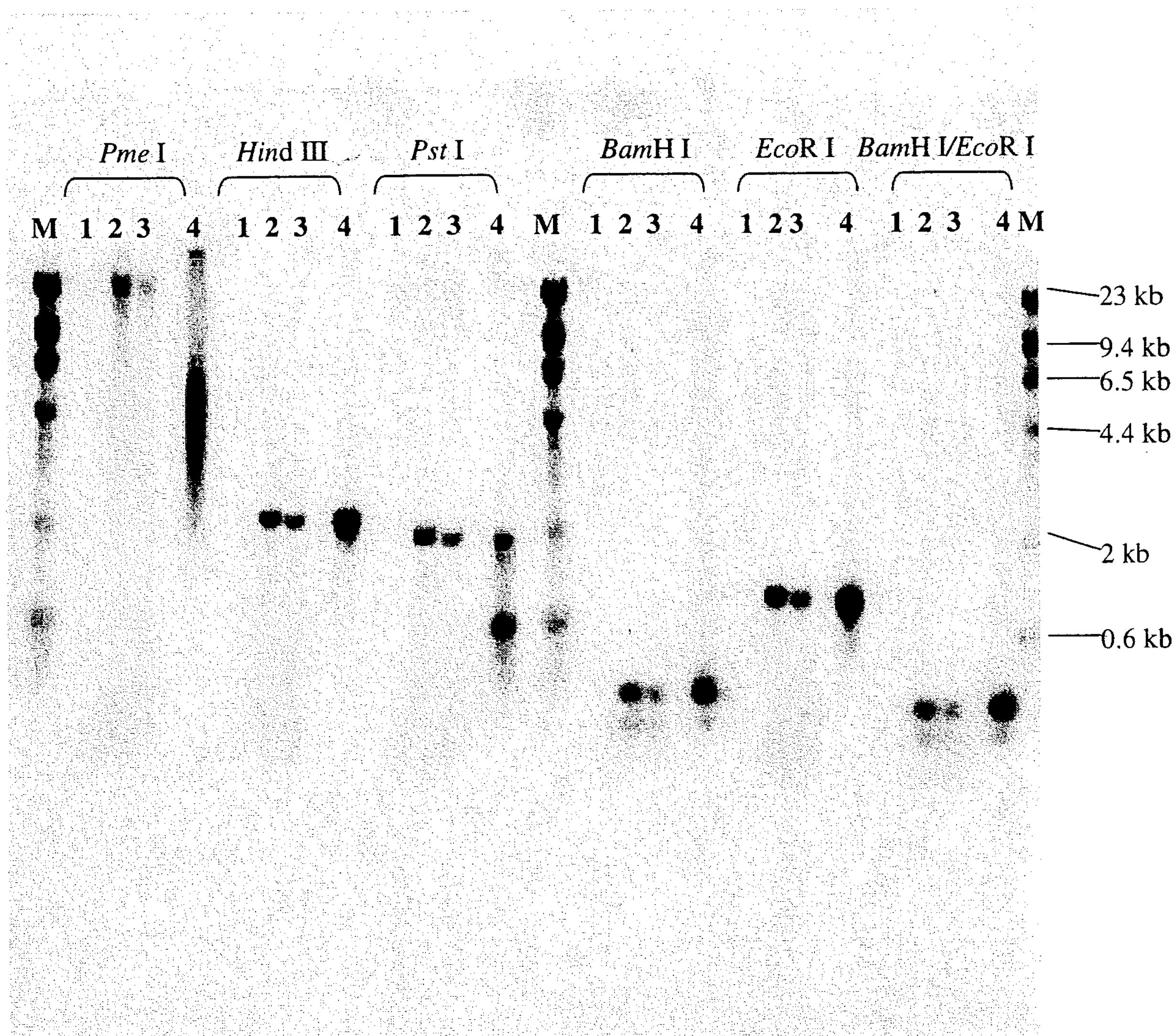
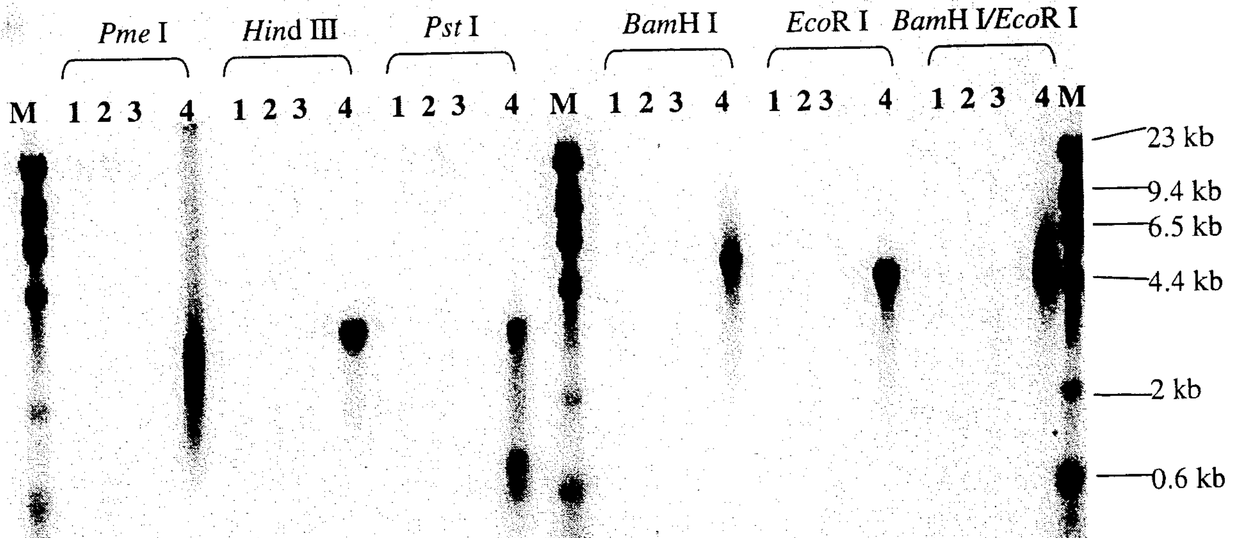
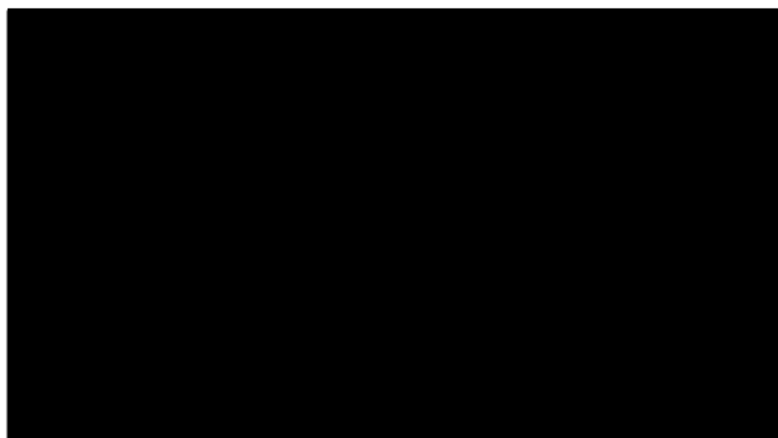


Figure 8: Southern analysis confirming the absence of the *nptII* gene in the DNA insert of maize line 1507.

DNA's were isolated from corn containing event 1507 and from the GS3 non-transgenic line. DNA's were digested with *Pme* I, *Hind* III, *Pst* I, *Bam*H I, *Eco*R I and *Bam*H I/*Eco*R I. The lanes contain:

1: GS3 non-transgenic control (5µg)
2: pooled T1S1 DNA (5µg)
3: pooled BC4 DNA (5µg)
4: plasmid PHP 8999 control (9.5 pg, equivalent to 1 copy)
M: molecular weight marker (*Hind* III digest of lambda DNA) (1 µg). MW of lambda DNA fragments is given in kilobases (kb).





2.3e Breeding Pedigree

Describe how the line or strain from which the food was obtained from the original transformant (i.e. give the family tree or describe the breeding process).

The *cry1*F and *pat* genes were introduced (transformed) into a line of maize called Hi-II. This line then became known as *B.t.*Cry1F maize line 1507 and its genetic makeup was 100% Hi-II. *B.t* Cry1F maize line 1507 was crossed to an elite inbred so the resulting progeny contained 50% Hi-II germplasm and 50% elite inbred germplasm. Based on Mendelian genetics only 50% of the progeny would contain the *cry1*F and *pat* genes and are referred to as the positive plants, the 50% of the progeny that did not contain the *cry1*F and *pat* genes are referred to as the "null segregants". The positive plants containing 50% Hi-II germplasm and 50% elite inbred germplasm are then crossed again (or backcrossed) to the elite inbred. The resulting progeny contain 25% Hi-II germplasm and 75% elite germplasm. This process is repeated until the percent elite germplasm is very close to 100%, and the *cry1*F and *pat* genes are also present in the elite inbred. Hybrid corn seed sold to farmers is produced by crossing two distinct inbred corn lines. Each inbred corn line has a different genetic background that allows the hybrid seed to be optimized for a specific geographical region where corn is grown. Seed companies may sell over 100 different hybrid seed products; therefore, these companies develop hundreds of inbred corn lines. A new gene, such as the *cry1*F gene in event TC1507, is introduced into the many different inbred lines through backcrossing.

2.3f Genetic Stability

Provide evidence of the stability of the genetic changes including:

- *the pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored*
- *the pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments*

Through backcrossing and selfing, as shown in Figure 9 and described below, it can be concluded that the *cry1*F and *pat* genes are genetically stable for at least six generations.

In addition, these results support the conclusion that maize line 1507 is genetically stable and that the *cry1*F and *pat* genes are inherited as Mendelian dominant genes. Results from Southern blot analysis show that the additional copy of the *cry1*F gene was present in the BC4 backcross generation (part 2.3d above) thus supporting the conclusion that it is genetically linked to the insert containing the *cry1*F and *pat* genes. The Southern analyses summarized in part 2.3d confirm that the results of restriction digests were similar for the T1S1 and BC4F1 generations, which provides further confirmation that the inserted DNA has been stably inherited in maize line 1507.

The original transformed Hi-II maize line containing event 1507 (transformant T0) was crossed to an elite inbred to give an F1 hybrid. The F1 hybrid was backcrossed twice to the elite inbred to give BC2F1 seed. Spraying at each generation eliminated plants susceptible to glufosinate-ammonium herbicide and resulted in seed hemizygous for the 1507 modification.

The seed from the BC2F1 generation was planted, and the plants were sprayed with glufosinate-ammonium. The expected segregation ratio was 1:1 (tolerant:susceptible) for glufosinate-ammonium tolerance (Figure 9). This is shown by the BC2F1 data in Table 5.

Table 5: Mendelian segregation of maize line 1507. Early segregation data obtained from the BC2F1 generation; later segregation data obtained from the F1 generation

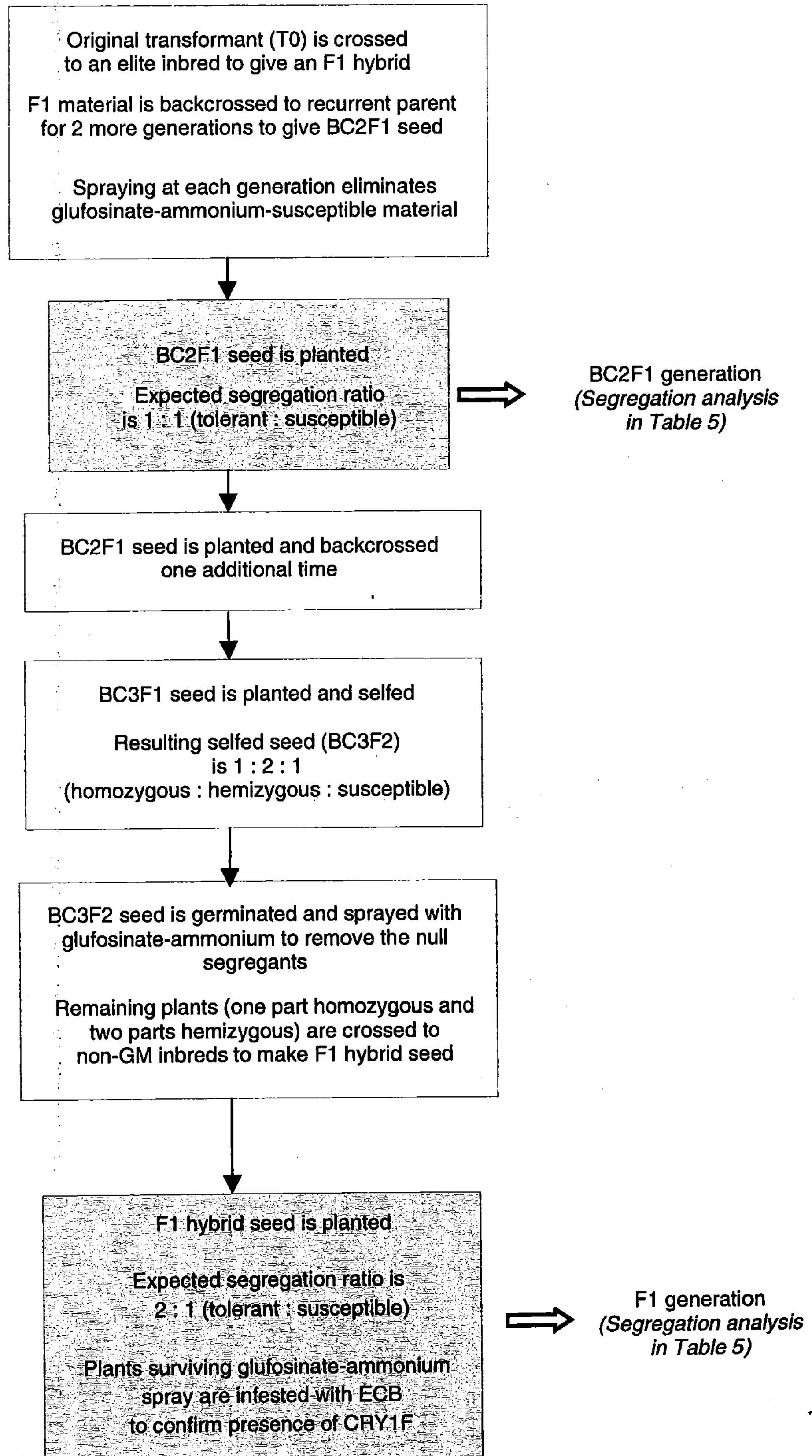
Generation	Observed ratio[a]	Expected ratio	Chi Square	p-value	Significant difference?[b]
BC2F1	248 : 278	263 : 263	1.711	0.1909	No
F1	910 : 493	935.3 : 467.7	2.903	0.0884	No

[a] Data expressed as number of observed tolerant to glufosinate-ammonium : number of observed plants susceptible to glufosinate-ammonium
[b] Significant at alpha = 0.05

Later segregation data were obtained from F1 hybrid seed as follows: after three backcrosses, maize line 1507 seed (BC3F1) was planted and self-pollinated. Resulting seed (BC3F2) was expected to be 3:1 (tolerant:susceptible) for glufosinate-ammonium tolerance. This seed was planted and sprayed with the herbicide to remove the homozygous susceptible plants. The remaining plants (one part homozygous tolerant and two parts hemizygous tolerant) were crossed to a susceptible inbred to make F1 hybrid seed. This hybrid seed was planted and sprayed with glufosinate-ammonium to check for the expected 2:1 (tolerant:susceptible) ratio (Figure9). This is shown by the F1 data in Table 5.

After the hybrids were sprayed with glufosinate-ammonium and scored for tolerance, 200 neonate European corn borers (ECB) were used to infest each F1 plant that survived the glufosinate-ammonium spraying. All of the plants determined to be tolerant to glufosinate-ammonium were also found to be resistant to European corn borer infestation.

Figure 9: Backcrossing generations used to determine Mendelian segregation ratios for maize line 1507



2.4 Antibiotic Resistance Genes

No antibiotic resistance genes were used during the transformation of maize line 1507.

2.4a Clinical Relevance

If applicable provide information on the clinical or veterinary importance in Australia and New Zealand of the antibiotic to which any transferred antibiotic resistance genes confer resistance.

2.4b End Use Viability (micro-organisms)

If the new GM organism is a microorganism, indicate whether it will remain viable in the final food.

2.4c Food Fraction DNA

Provide information on whether DNA is likely to be present in the food fractions derived from the new GM food (e.g. meal, refined oil, starches, etc).

2.5 Characterisation of the Novel Proteins

Field studies representing pertinent conditions and growth stages reflecting commercial production of maize were conducted during the 1998-1999 growing season in Chile, and during the 1999 growing season in France and Italy and the USA. The expression levels of CRY1F and PAT proteins in leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant tissues from maize line 1507 and a non-GM control with comparable background genetics were measured using specific Enzyme Linked Immunosorbent Assay (ELISA) developed for each protein. The results have been summarised below and full details provided in reports enclosed (Stauffer and Rivas 1999; Stauffer 2000){Zeph 2001 82 /id}

In addition, the characteristics of the newly expressed proteins in 1507 maize were studied by Western blot analyses. The specificity of expression of the *cry*1F and *pat* genes (novel genetic material) in maize line 1507 was confirmed.

Finally, further verification of the specificity of expression of novel genetic material has been provided by agronomic data collected from field trials in the USA (Stauffer and Rivas 1999), which show that maize line 1507 is comparable to non-GM maize, except for resistance to certain lepidopteran insect pests and tolerance to glufosinate-ammonium herbicide as derived from the genetic modification, and that no unexpected phenotypic differences are observed. The results are presented below.

2.5a Biochemical function and phenotypic effect of novel proteins

Provide a full description of the biochemical function and phenotypic effects of all novel proteins that could potentially be expressed in the new GM organism, including those resulting from the transfer of marker genes.

Maize line 1507 has been modified to express CRY1F protein for resistance to certain lepidopteran insect pests, such as the European corn borer (ECB), and to express phosphinothricin-N-acetyltransferase (PAT) protein for tolerance to glufosinate-ammonium herbicide. The *nptII* gene, conferring resistance to kanamycin, was not part of the purified linear DNA fragment used in the transformation and it is not present in maize line 1507, as confirmed by the molecular analyses carried out with Southern blots.,

Many natural δ-endotoxins are produced by *Bacillus thuringiensis* strains as insoluble parasporal crystalline inclusions and they are comprised of proteins (protoxins) approximately 120-140 kDa in size (Schnepf *et al.*, 1998). Upon ingestion by susceptible insects, these classes of protoxin crystals dissolve in the alkaline conditions of the insect gut and are processed by proteases to release the active core toxin comprised of the amino-terminal portion of the molecule. The activated toxins are typically 65-70 kDa in size. These toxins bind to specific receptors on the apical microvilli of epithelial midgut cells of insect pests. Binding of the activated toxin is followed by a conformational change of the toxin and its insertion into the membrane. Toxin oligomerization occurs with formation of pores in the cell membrane of the midgut cells causing osmotic cell lysis leading to insect death.

Cry1F is a 133.6 kDa protein with significant insecticidal activity against selected lepidopteran larvae. The spectrum of activity differs from other Cry1 proteins (Chambers *et al.* 1991).

The active ingredient in glufosinate-ammonium herbicide is L-phosphinothricin (L-PPT). L-PPT binds to glutamine synthetase in plants preventing the detoxification of excess ammonia resulting in plant death. The activity of the PAT protein (phosphinothricin-N-acetyltransferase) is specific to catalysing the conversion of L-PPT to N-acetyl-L-PPT. This is an inactive form which does not bind to glutamine synthetase (De Block *et al.* 1987). The expression of the PAT protein in maize line 1507 allows the detoxification of ammonia to continue and confers tolerance to the herbicide glufosinate-ammonium. The activity of the PAT protein has been described in detail by the OECD (1999): "Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide".

2.5b Novel Protein expression

Provide data of the level and site of expression of all novel proteins, particularly whether it is likely to be present in the edible portions of the organism, and if so, at what levels.

Expression of CRY1F and PAT proteins in maize line 1507 plant 3 studies

(1) Field study in Chile (1998-1999): (Stauffer and Rivas 1999) Ref 41 + 1

The study was conducted at four locations in the major maize growing regions of Chile. The locations were near the towns of Buin, Viluco, Graneros and Nancagua. These locations are comparable to regions in North America and Europe where the maize varieties would be suitable as commercial products.

All tissue samples were collected from a single replication of 1507 hybrid and control maize at each location. For the leaf sample, the youngest whorl leaf was collected from five plants when the plants were at approximately the V9 (or nine leaf) stage of development (Iowa State University, 1993). Pollen, silk and stalk samples were taken from five discrete plants at approximately the R1 stage (50% pollen shed) of development. Grain from five self-pollinated plants was collected after physiological maturity. The whole plant samples (entire plant except roots) consisted of three self-pollinated plants harvested at R4 stage (approximately four weeks after pollination), that were pooled at each location. The senescent whole plant samples, including ears, were harvested when the plant tissue had turned brown and dried. None of the plots were sprayed with glufosinate-ammonium.

The field studies demonstrate that expression of the CRY1F protein was found at measurable levels in all plant material sampled and tested of maize line 1507 (Table 6). Expression of the PAT protein was only found at measurable levels in the leaf tissue (V9 stage) samples of maize line 1507. The concentration of PAT in leaves of maize line 1507 was from below the limit of detection (LOD) to 40.8 pg/µg total extractable protein (TEP). As expected, expression of the CRY1F and PAT proteins was not detected in any samples from the non-GM control plants. The limits of detection were 10 pg/µg total extractable protein for CRY1F, and 20 pg/µg total extractable protein for PAT.

(2) Field study in France and Italy (1999): (Stauffer 2000) Ref 40

The study was conducted at three locations in France and three locations in Italy, all in the major maize growing regions of the EU. At each location in Italy, the trial consisted of 1507 maize hybrid sprayed with glufosinate-ammonium, 1507 maize hybrid unsprayed (without glufosinate-ammonium), and a non-GM control hybrid with genetics representative of 1507 maize. At each location in France, the trial involved 1507 maize hybrid unsprayed (without glufosinate-ammonium) and a non-GM control hybrid with genetics representative of 1507 maize. Not all samples were analysed for proteins as there were some losses due to mould contamination or other factors.

All tissue samples were collected from a single replication of 1507 maize and control maize. Leaf at V9 stage, whole plant at V9 stage, pollen, silk, stalk, whole plant at R1 stage, whole plant at R4 stage, grain and senescent whole plant tissue samples were collected at the different growth stages. Whole plant forage (R4 stage) and grain were collected from the glufosinate-ammonium sprayed plots as well. The samples were evaluated for CRY1F and PAT protein levels using specific ELISA methods developed for each protein.

The field studies show that expression of the CRY1F protein was found at measurable levels in all plant material sampled and tested (Table 7). The levels of expression of CRY1F found in the 1998/99 and 1999 trials were comparable for stalk, whole plant (R4 stage) and grain samples. The apparent differences observed in leaf, pollen and senescent whole plant can be explained by differences in the quality of the samples, which may have caused a decrease in total extractable protein content and thereby a relative increase in CRY1F content. In addition, the differences observed are not consistent with no indication of an obvious trend. For example, leaf expression levels of CRY1F were lowest in samples from Chile, but senescent whole plant samples showed lowest CRY1F levels in samples from the EU.

Expression of the PAT protein was found at measurable levels in the leaf tissue and whole plant (V9 stage) of 1507 maize. The concentration of PAT was from below the LOD to 136.8 pg/μg total extractable protein in leaves of 1507 maize, and from below the LOD to 38.0 pg/μg total extractable protein in whole plant tissue (V9 stage). As expected, expression of the CRY1F and PAT proteins was not detected in any samples from the non-GM control plants. The limits of detection were 10 pg/μg total extractable protein for CRY1F, and 20 pg/μg total extractable protein for PAT.

Field study in the USA (1999): (Zeph 2001)

A study was conducted at four locations in the midwestern maize growing region of the USA. Two of the locations were in Johnston, Iowa , one in Noblesville, Indiana and one in Windfall, Indiana.

Because the test lines were segregating for the *pat* and *cry1F* genes, it was necessary to identify positive plants that were expressing the PAT and Cry1F proteins. Since the two genes are genetically linked, plants that are tolerant to glufosinate-ammonium herbicide are also considered to be expressing the Cry1F protein. Therefore, the test lines were treated with glufosinate-ammonium herbicide by leaf painting plants at approximately the V4 to V5 stage of development. Plants that were damaged by the herbicide were assumed to lack the *pat* and *cry1F* genes and were removed from the plots.

All tissue samples were collected from a single replication of 1507 hybrid and control maize at each location. For the leaf sample, the youngest whorl leaf was collected from five plants when the plants were at approximately the V9 (or nine leaf) stage of development (Iowa State University, 1993). Pollen, silk and stalk samples were taken from five discrete plants at approximately the R1 stage (50% pollen shed) of development. Grain from five positive plants was collected after physiological maturity. The whole plant samples (entire plant except roots) consisted of three self-pollinated plants harvested at R4 stage (approximately four weeks after pollination), that were pooled at each location. The senescent whole plant samples, including ears, were harvested when the plant tissue had turned brown and dried.

Table 8 summarizes the Cry1F protein concentration measured in tissues collected from maize hybrid line 1507. Cry1F expression was highest in stalk tissue (1450 pg/μg TEP; mean) and lowest in silk tissue (37.4 pg/μg TEP; mean). The LOD for Cry1F protein was 10 pg/μg TEP. These levels of Cry1F protein expression are typical of Cry1F maize lines that exhibit effective control of European corn borer and Cry1F susceptible insect pests of maize. The results are similar to the expression levels found in other *B.t.* Cry1F maize hybrid lines (Stauffer and Rivas, 1999) where it was observed that levels of Cry1F protein expression were highest in whole plant and stalk tissues and lower in leaf, pollen and grain tissues. Cry1F protein expression was lowest in silk tissue in both studies. PAT protein was only expressed at measurable levels in leaf tissue and ranged from <LOD – 54.8 pg/μg TEP. In all other tissues, the levels of PAT protein were below the limit of detection (20 pg/μg TEP). The levels of PAT protein expression in maize leaves of the test hybrid were sufficient to confer tolerance to glufosinate-ammonium herbicide. Expression of the CRY1F and PAT proteins was not detected in any samples from the control plants.

Characteristics of the CRY1F and PAT proteins as expressed in maize line 1507

Western blot analytical techniques were applied to determine if the CRY1F and PAT proteins expressed in the maize line 1507 plants grown in Chile for the quantitative analysis above (Stauffer and Rivas 1999) were of the same molecular weight and immunoreactivity as the microbially-derived proteins (Alarcon and Marshall 2000). Polyclonal antibodies that recognize multiple antigenic epitopes on the protein were used. Any protein that is smaller (a partial protein) or larger (a fusion protein) in size than expected would be detected as a band of molecular weight that is different from the molecular weight of either the microbially-derived CRY1F or PAT protein.

Leaf, pollen, grain and whole plant tissues from field grown maize line 1507 plants were sampled for protein extraction. These tissues were originally obtained during the field studies conducted in the 1998/99 growing season in Chile as described above.

The results of the Western blot analyses of CRY1F protein expression in plant tissues from maize line 1507 are shown in Figure 10. The CRY1F protein was detected as two bands of approximately 65 to 68 kDa in leaf, pollen, whole plant and grain tissue. Expression of CRY1F protein was measurable in all four of these tissues as shown in Table 6. No other bands indicative of a partial CRY1F protein or a fusion protein of greater molecular weight were observed in maize line 1507 tissues. No immunoreactive proteins were detected in the negative control tissues, with the exception of a possible weakly reactive band in the negative control for grain tissue. Although this weakly reactive band is not readily visible in the Western blot (Figure 10), it has an apparent molecular weight that is slightly greater than the CRY1F protein, and is probably due to binding of the CRY1F antibody to an epitope present on an endogenous maize protein.

The CRY1F protein detected in Western blot analyses of maize line 1507 plant tissues was present as two bands of nearly identical molecular weight, commonly referred to as a "doublet." Protein doublets typically occur during gel electrophoresis if terminal amino acid residues have been removed from the protein as a result of the activity of proteases released during processing of the plant tissue for analysis. In a separate study, N-terminal amino acid sequence analysis of CRY1F protein derived from plant tissue showed that a five amino acid sequence corresponding to the expected N-terminus of proteolytically cleaved CRY1F was obtained (Evans 1998). The observed sequence was ^{28}STGRL (the superscript denotes the amino acid residue in the protein). This N-terminal sequence would be expected if cleavage of the CRY1F protein occurred during its purification from plant tissue due to the presence of trypsin-like enzyme activity (trypsin cleaves after arginine residues) (see Figure 3). The N-terminal sequence of the full-length 68 kDa CRY1F protein expressed *in planta* was blocked and therefore could not be sequenced. Hence, it appears that the doublet resulted from limited N-terminal processing by a plant protease with trypsin-like specificity.

The PAT protein is known to be a homodimer of approximately 43 kDa in the native form, and it is comprised of two components of approximately 22 to 23 kDa (OECD, 1999). The results of the Western blot analyses of PAT protein expression in plant tissues from maize line 1507 are shown in Figure 11. PAT protein was detected by Western blot analyses in leaf tissue of maize line 1507 as a band of approximately 22 kDa. No PAT protein was observed in pollen, whole plant or grain of maize line 1507. These results are consistent with the levels of PAT protein measured in maize line 1507 tissues. One additional band was observed to react with the PAT antibody in leaf tissue samples, but not in the negative control. This band may represent the ~43 kDa form of the protein that did not denature during gel electrophoresis. All other bands observed in pollen and grain tissue samples had corresponding bands of the same relative mobility in the negative control, indicating that the polyclonal antibody used to detect the PAT protein also recognized an epitope on a limited number of endogenous maize proteins. The PAT protein standard used in this study also contained bands corresponding to both the 22 kDa PAT protein and the ~43 kDa dimer that did not denature during gel electrophoresis. No other bands indicative of a partial PAT protein or a fusion protein of greater molecular weight were observed in maize line 1507 tissues.

Table 6: Summary of CRY1F protein levels (in pg/ µg total extractable protein) in tissue collected from maize line 1507 from field trials in Chile in 1998-1999

Tissue (growth stage)[a]	Mean[b] CRY1F (pg/µg TEP[c])	Standard deviation	Min/max range (pg/µg TEP)
Leaf (V9 stage)	110.9	27.2	56.6 – 148.9
Pollen (R1 stage)	135.5	13.5	113.4 – 168.2
Silk (R1 stage)	50.3	16.5	26.8 – 79.8
Stalk (R1 stage)	550.0	104.0	355.9 – 737.4
Whole plant (R4 stage)	1063.8	361.7	803.2 – 1572.7
Grain (Physiol. maturity)	89.8	23.3	71.2 – 114.8
Senescent whole plant (Brown and dry)	714.3	95.5	622.2 – 845.3

a: (Iowa State University 1993)
b: Values are means across all four sites from mean values calculated from the analysis of five individual samples per site for leaf, pollen, silk, stalk, grain and one pooled sample per site for both whole plant samples
c: TEP = total extractable protein

Table 7: Summary of CRY1F protein levels (in pg/µg total protein) in tissue collected from 1507 maize from field trials in France and Italy in 1999

Tissue (growth stage)[a]	Mean[b] CRY1F (pg/µg TEP[c])	Standard deviation	Min/max range (pg/µg TEP)
Leaf (V9 stage)	348.0	160.9	193.2 – 651.4
Whole plant (V9 stage)	743.7	394.2	409.6 – 1526.6
Pollen (R1 stage)	190.5	84.4	141.9 – 630.8
Silk (R1 stage)	133.0	58.1	61.1 – 265.3
Stalk (R1 stage)	630.8	141.6	417.9 – 917.7
Whole plant (R1 stage)	671.9	348.2	323.4 – 1206.4
Whole plant (R4 stage)	1073.1[d] 569.4[e]	338.2 11.0	874.4 – 1576.1 556.7 – 575.8
Grain (Physiol. maturity)	96.4[d] 90.3[e]	25.9 21.8	44.8 – 135.3 57.4 – 131.8
Senescent whole plant (Brown and dry)	198.9	21.4	171.2 – 219.5

a: (Iowa State University 1993)
b: Values are means across all sites. Samples were taken from plants not sprayed with glufosinate-ammonium unless stated otherwise
c: TEP = total extractable protein
d: Unsprayed plants (i.e. without glufosinate-ammonium)
e: Sprayed with glufosinate-ammonium

Table 8: CRY1F protein levels (in pg/µg total protein) in tissue collected from maize line 1507 from field trials in the USA in 1999

Tissue (growth stage)[a]	Mean[b] (pg/µg TEP[c])	Standard Deviation	Min/Max Range (pg/µg TEP)
Leaf (V9)	138	27.9	99.8 – 179
Pollen (R1)	126	9.79	103 – 145
Silk (R1)	37.4	12.2	13.8 – 67.9
Stalk (R1)	1450	326	1020 - 2160
Grain (R6)	116	20.3	86.6 – 168
Whole Plant (Forage: R4)	628	308	379 – 910
Whole Plant (Senescence)	328	105	217 – 442

a: (Iowa State University 1993)
b: Values are means across all four sites from mean values calculated from the analysis of five individual samples per site for leaf, pollen, silk, stalk, grain and one pooled sample per site for both whole plant samples
c: TEP = total extractable protein

Figure 10: Immunoreactivity of the CRY1F protein expressed in tissues of maize line 1507. Electrophoresis was conducted under denaturing conditions.

Lanes are labelled as follows: Leaf: Leaf tissue from two individual maize line 1507 plants (23 µg and 55 µg of protein, respectively). Leaf (-): Leaf tissue from non-GM control maize (41 µg of protein). Pollen: Pollen tissue from two individual maize line 1507 plants (41 µg and 61 µg of protein, respectively). Pollen (-): Pollen tissue from non-GM control maize (26 µg of protein). Whole plant: Whole plant tissue from two individual maize line 1507 plants (12 µg and 6 µg of protein, respectively). Whole plant (-): Whole plant tissue from non-GM control maize (14 µg of protein). Grain: Grain tissue from two individual maize line 1507 plants (82 µg and 98 µg of protein, respectively). Grain (-): Grain tissue from non-GM control maize (79 µg of protein). CRY1F protein: Purified microbially-expressed CRY1F protein. Molecular weight standards from 4 kDa to 250 kDa are indicated on the figure.

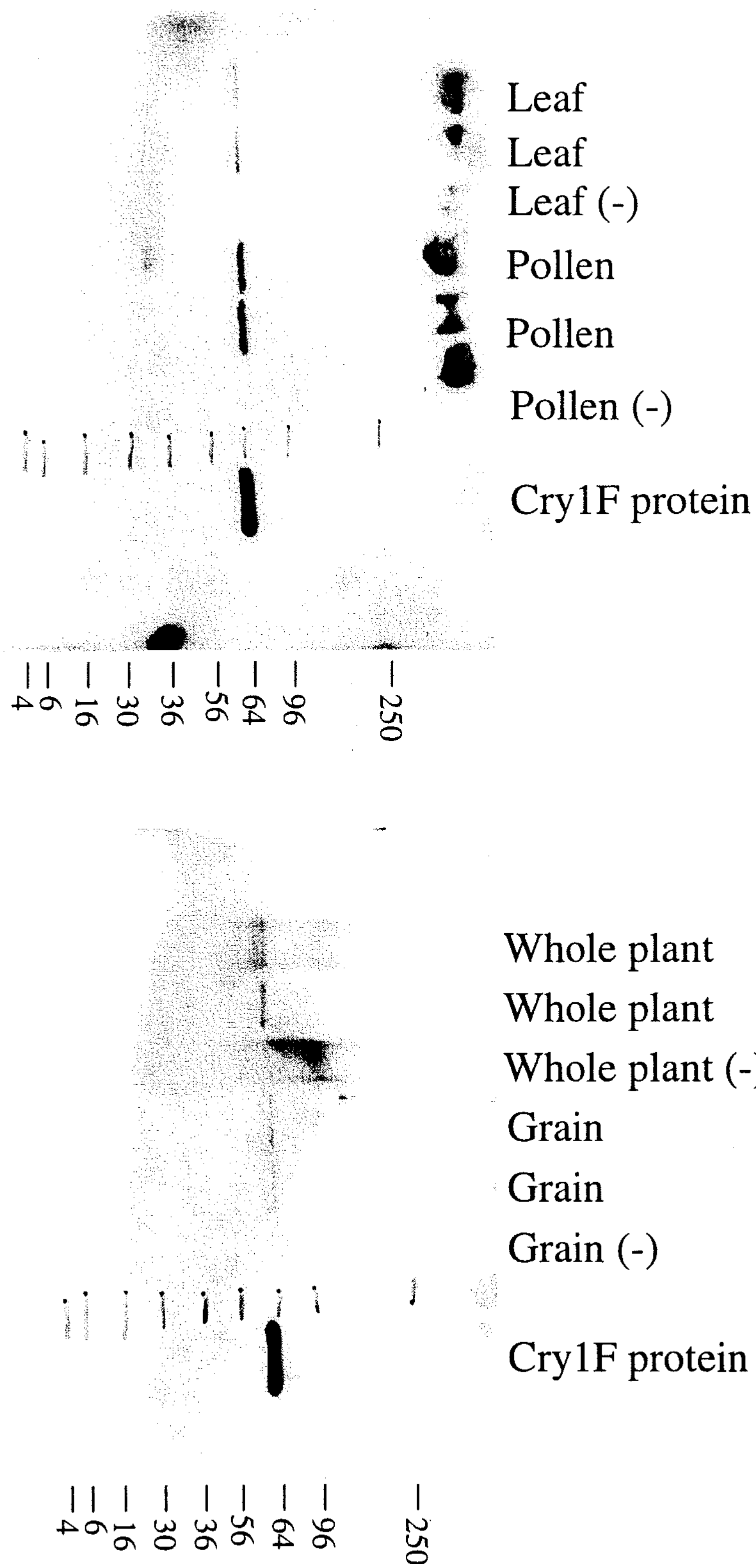
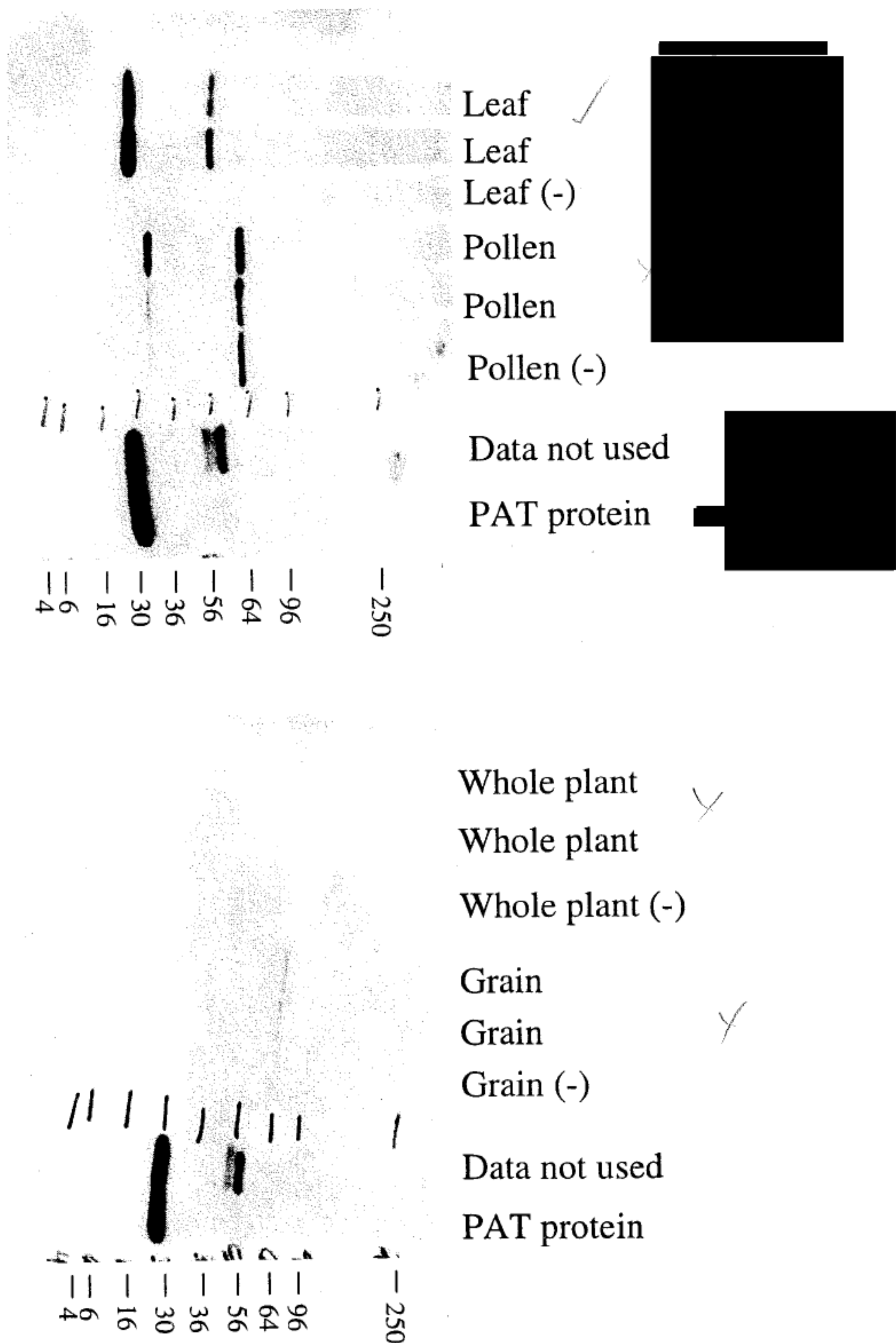


Figure 11: Immunoreactivity of the PAT protein expressed in tissues of maize line 1507. Electrophoresis was conducted under denaturing conditions.

Lanes are labelled as follows: Leaf: Leaf tissue from two individual maize line 1507 plants (23 µg and 55 µg of protein, respectively). Leaf (-): Leaf tissue from non-GM control maize (41 µg of protein). Pollen: Pollen tissue from two individual maize line 1507 plants (41 µg and 61 µg of protein, respectively). Pollen (-): Pollen tissue from non-GM control maize (26 µg of protein). Whole plant: Whole plant tissue from two individual maize line 1507 plants (12 µg and 6 µg of protein, respectively). Whole plant (-): Whole plant tissue from non-GM control maize (14 µg of protein). Grain: Grain tissue from two individual maize line 1507 plants (82 µg and 98 µg of protein, respectively). Grain (-): Grain tissue from non-GM control maize (79 µg of protein). PAT protein: Microbially-expressed PAT protein. Molecular weight standards from 4 kilodaltons (kDa) to 250 kDa are indicated on the fiqure.



2.5c Novel Protein silencing

In the case where a transferred gene is not likely to express any novel protein (e.g. because it has been "silenced" or is in a non-functional form), evidence of non-expression should be provided.

Neither of the two genes transferred to the maize lines have been silenced through mechanisms such as gene co-suppression. Note that the *nptII* gene was discarded after digestion of the plasmid provided the 6235 bp insert containing the plant optimized and truncated *cry*1F gene and the plant optimized *pat* gene plus the necessary regulatory sequences.

2.5d Novel protein history of consumption

Provide information about any prior history of human consumption of the novel protein, or its similarity to proteins that have been previously consumed in food.

Maize as the recipient plant, and donor of the *ubi*ZM1(2) promoter, is not a pathogenic organism and has a long history of domestication and safe use as an agricultural food crop.

Bacillus thuringiensis, donor of the *cry*1F sequence, has a history of decades of safe use as a pesticide (EPA 1996). The subspecies *aizawai* is commercially used to control wax moth larvae and various caterpillars, especially the diamondback moth caterpillar (Cornell University 1996).

Agrobacterium tumefaciens is the source of the ORF25PolyA terminator for the *cry*1F gene. No sequences involved in plant pathogenicity are encoded by this transcription terminator.

Streptomyces viridochromogenes, donor of the *pat* gene, is a common soil bacterium that produces the tripeptide L-phosphinothricyl-L-alanyl-alanine (L-PPT), which was developed as a non-selective herbicide by Hoechst Ag. There is a history of safe use of the *pat* gene in GM crops (OECD 1999).

The cauliflower mosaic virus, donor of the CaMV 35S promoter and terminator sequences, is a DNA caulimovirus with a host range restricted primarily to cruciferous plants (ICTV Database 1998).

2.5e Acute oral toxicity studies

Provide the results of any acute oral toxicity studies that have been conducted with the novel proteins, particularly where the protein does not have a prior history of safe human consumption.

The potential toxicity of the maize expressed core CRY1F protein to humans and animals was examined in an acute oral toxicology study where the equivalent microbially derived core CRY1F protein was evaluated for acute toxicity potential in mice (Kuhn 1998). The highest dose tested was 5050 mg of test material per kg body weight. When adjusted for purity of the test material (11.4%) (Evans 1998), the dose was 576 mg CRY1F per kg body weight. Observations for mortality and/or clinical or behavioural signs of pathology as well as body weights were made during the course of the study, and gross necropsies were conducted at the end of the study. No mortality occurred during the course of the study. Additionally, no adverse clinical signs were observed during the study and no adverse findings were noted at necropsy. The relatively high dose tested in this study did not give rise to any toxicity and therefore the acute LD_{50} for CRY1F protein could not be determined other than estimated to be higher than 576 mg CRY1F per kg body weight.

The safety in terms of toxicity for the PAT protein has already been determined in detail during the assessment of glufosinate-ammonium tolerant maize (Canadian Food Inspection Agency 1998; EPA 1995; EPA 1997; OECD 1999; Scientific Committee on Plants 1998). The *pat* gene was originally obtained from *Streptomyces viridochromogenes* strain Tü494 which has no known toxic or pathogenic potential. The PAT protein is enzymatically active but it has high substrate specificity to the active ingredient of glufosinate-ammonium (L-PPT), and such a substrate does not occur within the maize plant or within the animal and human diets.

An acute oral toxicity study consisting of feeding mice with test material containing approximately 5000 mg PAT per kg body weight was carried out (Brooks 2000). There were no treatment-related clinical observations. All the mice in the study, 5 males and 5 females, gained body weight normally over the two-week observation period with no gross pathologic lesions. Due to the absence of any

symptoms of toxicity in this study, the LD_{50} of PAT protein could not be determined other than estimated to be higher than 5000 mg PAT per kg body weight.

2.5f Comparative amino acid sequence with toxins

Provide a comparison of the amino acid sequence of each of the novel proteins to known protein toxins.

No data are provided comparing amino acid sequences on the novel proteins to known toxins. The toxicity tests and history of safe use discussed above indicates that the novel proteins are not toxic to humans.

2.5g Immunochemical reactivity - suspected allergens

In the novel protein is from a source known to be allergenic provide information on the immunochemical reactivity of the introduced protein with IgE from the blood serum of affected individuals. If these results are negative, skin tests and double blind oral food challenges should be conducted as confirmation.

The most important factor to consider in assessing allergenic potential is whether the source of the gene being introduced into plants is known to be allergenic (FDA 1992). Neither *Bacillus thuringiensis* (the source of the *cry*1F gene) nor *Streptomyces viridochromogenes* (the source of the *pat* gene) have a history of causing allergy. Also, both donor organisms are common soil bacteria. In over 30 years of commercial use, there have been no substantiated reports of allergenicity to *B. thuringiensis,* including occupational allergy associated with manufacture of products containing *B. thuringiensis* (EPA 1995; EPA 1996; McClintock *et al.* 1995). Therefore no immunochemical reactivity assays have been conducted with these proteins.

2.5h Comparative amino acid sequence with allergens

If the novel protein is from a source not known to be allergenic, provide information on:

- *the amino acid sequence similarity of the novel protein with known allergens; and*
- *the stability of the novel protein to degradation in simulated gastric and intestinal fluids*

The biochemical profile of the CRY1F and PAT proteins provides a basis for allergenic assessment when compared with known protein allergens. A comparison of the amino acid sequence of an introduced protein with the amino acid sequences of known allergens is a useful indicator of allergenic potential (FAO/WHO 2000). A database search was compiled by Meyer (1999) using the Wisconsin Genetics Computer Group (GCG) sequence analysis computer program with the keyword "allergen" to search standard DNA and protein sequence databases. A significant homology is defined as a sequence identity of 8 or more contiguous amino acids. Comparison of the 15 most homologous database sequences confirmed that CRY1F protein does not share any significant amino acid sequence homology with known protein allergens.

In a similar way, a comparison of the amino acid sequence of PAT protein to known protein allergens was also carried out (Meyer 1999). As expected, the results demonstrated that PAT protein shares no significant amino acid homology with known protein allergens. In addition, the PAT protein has been the subject of previous safety assessments for genetically modified plants and found to have no potential for allergenicity (Canadian Food Inspection Agency 1998; EPA 1995; EPA 1997; Health Canada 1997; OECD 1999; Scientific Committee on Plants 1998).

Protein allergens are typically stable to the peptic and tryptic digestion, and to the acid conditions of the human digestive system, which allows them to reach and pass through the intestinal mucosa to elicit an allergenic response. Both CRY1F and PAT proteins are readily degradable in simulated digestive juice, minimising any potential for these proteins to be absorbed by the intestinal mucosa when consumed. The core CRY1F protein was nearly completely proteolysed in simulated gastric conditions within one minute at a molar ratio of 188:1 (CRY1F:pepsin) (Evans 1998). In addition, the immunoblot detection technique has demonstrated that CRY1F is not glycosylated, which is an additional indicator of the absence of allergenic potential in the CRY1F protein. Furthermore, CRY1F looses immunoreactivity after heat processing and it has a history of safe use in microbial pesticides (Evans 1998). In addition, a study on heat lability of core CRY1F protein at various temperatures has shown that CRY1F looses biological activity against neonate tobacco budworm, *Heliothis virescens*, after exposure at 75 $^{\circ}C$ or greater for 30 minutes (Herman and Korjagin 1999). The PAT protein degraded to non-detectable levels within 5 seconds after introduction to simulated gastric fluid containing pepsin (Glatt 1999; OECD 1999).

Therefore, the *cry*1F and *pat* genes introduced into maize line 1507 do not encode for known allergens, and neither the CRY1F nor the PAT proteins share immunologically significant amino acid sequences with known allergens. This together with the rapid breakdown of these proteins under digestive conditions, supports the conclusion that the CRY1F and PAT proteins do not pose any significant allergenic risk to humans.

2.6 Characterisation of other Novel Substances

2.6a Identity of other novel substances

Identify any other novel substances, (e.g.. metabolites) that might accumulate on or in the GM organism as a result of the genetic modification and determine their levels and site of accumulation.

Maize is not considered to have harmful toxicants and the genetic modification in maize line 1507 does not introduce any new toxicants harmful to humans, as discussed in 2.5e above. In addition, and as shown by the compositional analyses presented in 2.7 below, the levels of existing potential anti-nutrients in maize line 1507 are comparable to those of traditionally-bred (non-GM) maize.

2.6b Potential toxicity

If any other novel substances are identified, determine their potential toxicity.

No novel substances have been identified.

2.7 Comparative Analysis

2.7a Key nutrients

The levels of key nutrients, toxicants and anti-nutrients in the GM organism should be compared (using appropriate statistical analyses) with levels in the non-GM counterpart. Information about the range of natural variation should be provided for each constituent measured. Particular attention should be paid to those constituents that are present in the edible part of the organism, or that remain in the final food. If any of the levels are significantly different from those in the comparator and are outside the range of natural variation, additional studies may be required.

Food products derived from maize line 1507 are nutritionally equivalent to those derived from other commercial maize. Composition analyses of grain from maize line 1507 have shown that the contents of protein, fiber, carbohydrates, fat, ash, minerals, fatty acids, amino acids, vitamins, secondary metabolites and anti-nutrients are all equivalent to that found in non-GM maize with comparable genetic background and to the published range of values in the literature. In addition, nutritional equivalence between maize line 1507 and traditionally-bred maize has been shown in a poultry feeding study (Zeph 2000).

Specific studies on the compositional analyses of grain from hybrids of maize line 1507 and the traditionally-bred non-GM maize have been carried out. Composition data was obtained from trials carried out in Chile in 1998-1999. The methods used and the results from these analyses are presented below.

<u>Composition data from 1998-1999 (Chile)</u>

Compositional analyses was carried out on grain from maize line 1507 and from a non-GM control maize with comparable genetics (Stauffer and Rivas 1999; Stauffer and Zeph 2000).
Ref 42

The field trials were conducted at four locations in the major maize growing regions of Chile. The locations were near the towns of Buin, Viluco, Graneros and Nancagua. These locations are comparable to regions of North America and Europe where the maize varieties would be suitable as commercial products. Therefore the nutritional composition of grain should be comparable to composition of grain from similar locations in North America and Europe.

At each location, there were six replicates of maize line 1507 and control maize. The analyses of grain from each replicate consisted of protein, fiber, carbohydrates, fat, ash, minerals, fatty acids, amino acids, vitamins, secondary metabolites and anti-nutrients. These analyses were conducted at Woodson-Tenent Laboratories according to the methods of the Association of Official Analytical

Chemists (Helrich 1990).

i) Proximate analyses of maize grain

The grain was harvested at between 11-13 % moisture. Analyses to determine the level of protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), carbohydrate, fat and ash in grain from maize line 1507 and the non-GM control maize were carried out. Levels of these components in maize line 1507 grain and control maize were comparable and they were also within the literature range for maize grain (Table 9). No statistically significant differences were observed between the means for maize line 1507 and control maize, with the exception of total fat content: the difference in fat content was very small and within the range published in the literature, hence unlikely to be of any nutritional significance. Therefore, the analyses of proximate composition of grain from maize line 1507 showed that it is comparable to grain from control maize.

ii) Mineral analyses of maize grain

Levels of five minerals (calcium, magnesium, phosphorous, potassium and sodium) were analysed in maize line 1507 and control maize (Table 10). The levels of all these minerals were comparable and within the expected range for maize grain. In the case of calcium, comparisons to data obtained from analyses of 22 commercial Pioneer® Brand hybrids were included in addition to the ranges in the literature. The modifications made by Woodson-Tenent to the method to determine calcium levels typically results in lower amounts of calcium than those reported in the literature. Therefore, the comparison to the Pioneer hybrids, analysed by the same method, is more appropriate.

iii) Fatty acid analyses of maize grain

Five major fatty acids in grain from maize line 1507 and the control maize were analysed: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) (Table 11). The levels of all five fatty acids in maize line 1507 and control maize were comparable and within the literature range for maize grain.

iv) Amino acid analyses of maize grain

Eighteen amino acids were analysed in grain from maize line 1507 and the control maize (Table 12). The levels of all the amino acids in maize line 1507 and control maize were comparable and, with the exception of threonine and glutamic acid, within the range published in the literature or as determined by the analyses of Pioneer hybrids. There was no statistically significant difference in threonine or glutamic acid levels between maize line 1507 and control, indicating that the different levels of threonine and glutamic acid in maize line 1507 and control maize compared to the literature range was not due to the genetic modification.

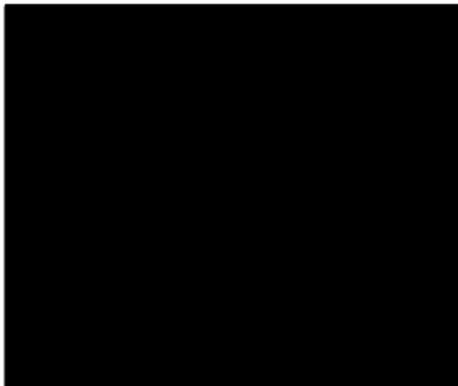
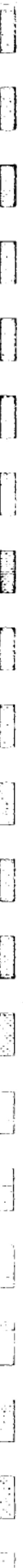
v) Vitamin analyses of maize grain

Grain from maize line 1507 and the control maize was analysed for content of four vitamins (Table 13). Vitamin B1, vitamin B2, total tocopherols and folic acid levels were determined for comparison to the available range of values for maize in the literature. Levels of vitamins B1, B2 and tocopherols in maize line 1507 were found to be comparable and within the available range of literature values for maize grain. There is no typical range available for folic acid in maize grain, although an average value of 0.3 ppm is reported (Watson, 1987). Levels of folic acid in maize line 1507 and the control maize were not significantly different.

vi) Secondary metabolites and anti-nutrient analyses of maize grain

Two potential anti-nutrients in maize, phytic acid and trypsin inhibitor, were analysed in grain from maize line 1507 and control maize (Table 14). Phytic acid levels in maize line 1507 were found to be within the published literature range for maize grain. Maize contains very low levels of the anti-nutrient trypsin inhibitor (Del Valle *et al.* 1983; Watson 1987). The trypsin inhibitor levels in both maize line 1507 and control maize were below the limit of quantitation (2000 TIU/g) of the enzyme assay that was used in these analyses. This confirms that no unusually high levels of trypsin inhibitor are present in maize line 1507.

<u>Composition data from 1999 (France and Italy)</u>

France / Italy study.

The field trials were carried out at three locations in France and three locations in Italy, all within the major maize growing regions in these countries. At each location in France, the trials involved six replicates of 1507 maize unsprayed with glufosinate-ammonium, and a non-GM control maize with comparable genetics. At each location in Italy, the trials involved three replicates of 1507 maize sprayed with glufosinate-ammonium, 1507 maize unsprayed with glufosinate-ammonium, and of a non-GM control maize with comparable genetics (Stauffer 2000). Ref 40

At each location, grain samples were taken from sprayed and unsprayed plots according to the treatments and number of replicates. The analyses of grain from each replicate consisted of protein, fiber, carbohydrates, fat, ash, minerals, fatty acids, amino acids, vitamins, secondary metabolites and anti-nutrients. These analyses were conducted at Woodson-Tenent Laboratories according to the (Helrich 1990).

i) Proximate analyses of maize grain

The grain was harvested at between 9-12 % moisture. Analyses to determine the level of protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), carbohydrate, fat and ash in grain of 1507 maize and the control maize were carried out. Levels of these components in 1507 maize and the control maize were comparable and no statistically significant differences were observed between 1507 maize and control maize (Table 15). With the exception of ADF, the levels of protein, NDF, carbohydrates, fat and ash were all within the literature range for maize grain of 1507 maize and control maize. Levels of ADF in 1507 maize and control maize were all slightly lower than the published range. However, there was no statistically significant difference in ADF levels between 1507 maize and control maize, indicating that the slightly lower levels in 1507 maize was not due to the genetic modification or spraying with glufosinate-ammonium.

Table 20

ii) Mineral analyses of maize grain

Levels of five minerals (calcium, magnesium, phosphorous, potassium and sodium) were analysed in 1507 maize and the control maize (Table 16). The levels of all these minerals were comparable and within the literature range, confirming that 1507 maize grain (unsprayed or sprayed with glufosinate-ammonium herbicide) was comparable to control maize in mineral composition. In the case of calcium, comparisons to data obtained from analyses of 22 commercial Pioneer® Brand hybrids were included in addition to the ranges in the literature. The modifications made by Woodson-Tenent to the method to determine calcium levels typically results in lower amounts of calcium than those reported in the literature. Therefore, the comparison to the Pioneer hybrids, analysed by the same method, is more appropriate.

iii) Fatty acid analyses of maize grain

Five major fatty acids in grain from 1507 maize and the control maize were analysed: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) (Table 17). The levels of all five fatty acids in 1507 maize (unsprayed or sprayed with glufosinate-ammonium herbicide) and control maize were comparable and within the literature range for maize grain.

iv) Amino acid analyses of maize grain

Eighteen amino acids were analysed in grain from 1507 maize and the control maize (Table 18). The levels of all the amino acids in 1507 maize and control maize were comparable and, with the exception of threonine and glutamic acid, within the range published in the literature or as determined by the analyses of Pioneer hybrids. Levels for threonine and glutamic acid in 1507 unsprayed and sprayed maize were slightly above the published ranges, but this is typical of the genetic background of this maize and is not considered of any nutritional significance. In general, the amino acid levels for unsprayed and sprayed 1507 maize were greater than those of the control maize, however this was not a trend observed for all nutrient composition traits. Additionally, the amino acid levels for control maize were lower than in the previous study carried out in Chile with the same hybrid, but the levels in 1507 maize were comparable. These small changes in levels of amino acids should not have a detrimental impact on the nutritional quality of maize.

v) Vitamin analyses of maize grain

Grain from 1507 maize and the control maize was analysed for content of four vitamins (Table 19).

Vitamin B1 and vitamin B2 levels were found to be comparable and within the available range of literature values for maize grain. There is no typical range available for folic acid in maize grain, although an average value of 0.3 ppm is reported (Watson 1987). Levels of folic acid in 1507 maize and control maize were not significantly different. There was no statistically significant difference in total tocopherols between 1507 maize and control maize, but they were all lower than the literature ranges. Tocopherols are rapidly degraded and the storage times and conditions of these samples may have resulted in an overall loss of tocopherol.

vii) Conclusion

Overall, the analyses of nutrient composition of grain (Chile, France and Italy) from 1507 maize grown at a range of locations over a period of two years show that grain from 1507 maize is comparable and nutritionally equivalent to grain from existing commercial maize hybrids. Furthermore, spraying with glufosinate-ammonium does not have an effect on the nutrient composition of grain from 1507 maize.

Table 9: Mean values of proximate composition of grain (Chile, 1998-1999)

Response variable[a]	1507	Non-GM control	Published range of values[b]
Protein %	11.20	11.32	6.0 – 12
ADF %	3.55	3.68	3.0 – 4.3
NDF %	10.47	10.08	8.3 – 11.9
Carbohydrates[c] %	83.45	83.23	63.3 – 89.7
Fat %	3.83	3.94	3.1 – 5.7
Ash %	1.51	1.50	1.1 – 3.9

a: Percentage on a dry weight basis
b: (Watson 1982)
c: Fiber (ADF and NDF) is included in the carbohydrates

Table 10: Mean values of mineral composition of grain (Chile, 1998-1999)

Response variable[a]	1507	Non-GM control	Published range of values
Calcium %	0.0036	0.0031	0.01 – 0.10[b] 0.002 – 0.011[c]
Magnesium %	0.12	0.13	0.09 – 1.0[b]
Phosphorous %	0.33	0.32	0.26 – 0.75[b]
Potassium %	0.40	0.36	0.32 – 0.72[b]
Sodium %	<LOQ[d]	<LOQ[d]	0.0 – 0.15[b]

a: Percentage on a dry weight basis
b: (Watson 1982)
c: Data from analyses of 22 commercial Pioneer® Brand Hybrids
d: Below the level of quantitation for sodium of 0.01%

Table 11: Mean values of fatty acid composition of grain (Chile, 1998-1999)

Response variable[a]	1507	Non-GM control	Published range of values[b]
Palmitic	11.07	10.92	7 - 19
Stearic	2.28	2.44	1 - 3
Oleic	30.61	32.53	20 - 46
Linoleic	53.10	51.16	35 - 70
Linolenic	1.29	1.21	0.8 - 2

a: Value of fatty acids is % of total lipid
b: (Watson 1982)

Table 12: Mean values of amino acid composition of grain (Chile, 1998-1999)

Response variable[a]	1507	Non-GM control	Published range of values[b]
Glycine	0.39	0.40	0.26 - 0.47[b] 0.24 - 0.41[c]
Threonine	0.40	0.41	0.29 - 0.39[b] 0.21 – 0.37[c]
Valine	0.51	0.52	0.21 - 0.52[b] 0.25 - 0.67[c]
Isoleucine	0.40	0.40	0.26 - 0.40[b] 0.19 - 0.39[c]
Leucine	1.42	1.43	0.78 -1.52[b] 0.43 - 1.35[c]
Phenylalanine	0.56	0.57	0.29 - 0.57[b] 0.04 - 0.54[c]
Histidine	0.29	0.30	0.20 - 0.28[b] 0.21 - 0.32[c]
Lysine	0.32	0.32	0.20 - 0.38[b] 0.19 - 0.36[c]
Arginine	0.44	0.45	0.29 - 0.59[b] 0.28 - 0.55[c]
Cysteine	0.21	0.23	0.12 - 0.16[b] 0.13 - 0.27[c]
Methionine	0.19	0.20	0.10 - 0.21[b] 0.12 - 0.26[c]
Tryptophan	0.08	0.08	0.05 - 0.12[b] 0.05 - 0.10[c]
Serine	0.54	0.55	0.42 - 0.55[b] 0.25 - 0.46[c]
Alanine	0.84	0.85	0.64 - 0.99[b] 0.37 - 0.81[c]
Glutamic acid	2.14	2.18	1.24 - 1.96[b] 0.89 - 2.02[c]
Proline	1.01	1.03	0.66 - 1.03[b] 0.43 - 1.01[c]
Aspartic acid	0.77	0.81	0.58 - 0.72[b] 0.37 - 0.80[c]
Tyrosine	0.20	0.20	0.29 - 0.47[b] 0.17 - 0.31[d]

a: Percentage on a dry weight basis
b: (Watson 1982)
c: Data from analyses of 22 commercial Pioneer® Brand Hybrids
d: (Iowa Department of Agriculture and Land Stewardship 2001)

Table 13: Mean values of vitamin composition of grain (Chile, 1998-1999)

Response variable[a]	1507	Non-GM control	Published range of values[b]
Thiamine hydrochloride (B1)	3.64	4.06	3.0 – 8.6
Riboflavin (B2)	1.67	1.66	0.25 – 5.6
Folic acid	0.151	0.144	0.3[c]
Total tocopherols	48.2	41.6	42 - 87

a: Parts per million (ppm) on a dry weight basis
b: (Watson 1982)
c: (Watson 1987) reports an average value for folic acid in grain as 0.3 mg/kg

Table 14: Mean values of anti-nutrient composition of grain (Chile, 1998-1999)

Secondary metabolites in grain			
Response variable[a]	**1507**	**Non-GM control**	**Published range of values[b]**
Inositol mg/100 g	59.96	61.68	NA
Raffinose %	0.086	0.087	0.08 – 0.30
p-Coumaric acid mg/100 g	16.5	16.0	NA
Furfural mg/100g	<LOQ[c]	<LOQ[c]	NA
Ferulic Acid mg/100 g	264	257	NA
Anti-nutrient analyses of grain			
Phytic acid %	0.99	0.96	0.7 – 1.0
Trypsin inhibitor TIU/g	<LOQ[d]	<LOQ[d]	NA

a: Percentage (%) or mg/100g on a dry weight basis. Trypsin inhibitor is expressed in units of trypsin inhibitor enzyme activity per gram on a dry weight basis
b: (Watson 1982)
c: Below level of quantitation for furfural of 0.500 mg/100 g
d: Below level of quantitation for trypsin inhibitor of 2000 TIU/g
NA = ranges are not available in the published literature

Table 15: Mean values of proximate composition of grain (France and Italy, 1999)

Response variable[a]	1507 (unsprayed)[b]	1507 (sprayed)[b]	Non-GM control	Published range of values[c]
Protein	11.73	12.04	10.98	6.0 – 12
ADF	2.37	2.52	2.29	3.0 – 4.3
NDF	10.16	10.54	10.13	8.3 – 11.9
Carbohydrates[d]	82.46	81.97	83.00	63.3 – 89.7
Fat	4.21	4.41	4.41	3.1 – 5.7
Ash	1.60	1.67	1.56	1.1 – 3.9

a: Percentage on a dry weight basis
b: Unsprayed or sprayed with glufosinate-ammonium herbicide
c: (Watson 1982)
d: Fiber (ADF and NDF) is included in the carbohydrates

Table 16: Mean values of mineral composition of grain (France and Italy, 1999)

Response variable[a]	1507 (unsprayed)[b]	1507 (sprayed)[b]	Non-GM control	Published range of values
Calcium	0.008	0.007	0.007	0.01 – 0.10[c] 0.002 – 0.011[d]
Magnesium	0.114	0.117	0.106	0.09 – 1.0[c]
Phosphorous	0.33	0.34	0.31	0.26 – 0.75[c]
Potassium	0.416	0.417	0.380	0.32 – 0.72[c]
Sodium	0.0015	0.0015	0.0015	0.0 – 0.15[c]

a: Percentage on a dry weight basis
b: Unsprayed or sprayed with glufosinate-ammonium herbicide
c: (Watson 1982)
d: Data from analyses of 22 commercial Pioneer® Brand Hybrids

Table 17: Mean values of fatty acid composition of grain (France and Italy, 1999)

Response variable[a]	1507 (unsprayed)[b]	1507 (sprayed)[b]	Non-GM control	Published range of values[c]
Palmitic	9.87	9.85	9.83	7 - 19
Stearic	2.09	2.03	2.11	1 - 3
Oleic	33.06	33.21	32.63	20 - 46
Linoleic	51.38	51.42	51.90	35 - 70
Linolenic	1.16	1.17	1.17	0.8 - 2

a: Value of fatty acids is % of total lipid
b: Unsprayed or sprayed with glufosinate-ammonium herbicide
c: (Watson 1982)

Table 18: Mean values of amino acid composition of grain (France and Italy, 1999)

Response variable[a]	1507 (unsprayed)[b]	1507 (sprayed)[b]	Non-GM control	Published range of values
Glycine	0.41	0.42	0.38	0.26 - 0.47[c] 0.24 - 0.41[d]
Threonine	0.41	0.41	0.37	0.29 - 0.39[c] 0.21 – 0.37[d]
Valine	0.51	0.52	0.47	0.21 - 0.52[c] 0.25 - 0.67[d]
Isoleucine	0.41	0.41	0.36	0.26 - 0.40[c] 0.19 - 0.39[d]
Leucine	1.38	1.41	1.23	0.78 -1.52[c] 0.43 - 1.35[d]
Phenylalanine	0.55	0.56	0.49	0.29 - 0.57[c] 0.04 - 0.54[d]
Histidine	0.31	0.32	0.29	0.20 - 0.28[c] 0.21 - 0.32[d]
Lysine	0.32	0.33	0.31	0.20 - 0.38[c] 0.19 - 0.36[d]
Arginine	0.47	0.48	0.44	0.29 - 0.59[c] 0.28 - 0.55[d]
Cysteine	0.22	0.23	0.22	0.12 - 0.16[c] 0.13 - 0.27[d]
Methionine	0.20	0.21	0.20	0.10 - 0.21[c] 0.12 - 0.26[d]
Tryptophan	0.10	0.10	0.09	0.05 - 0.12[c] 0.05 - 0.10[d]
Serine	0.55	0.56	0.50	0.42 - 0.55[c] 0.25 - 0.46[d]
Alanine	0.83	0.85	0.74	0.64 - 0.99[c] 0.37 - 0.81[d]
Glutamic acid	2.12	2.18	1.90	1.24 - 1.96[c] 0.89 - 2.02[d]
Proline	1.00	1.04	0.92	0.66 - 1.03[c] 0.43 - 1.01[d]
Aspartic acid	0.79	0.81	0.71	0.58 - 0.72[c] 0.37 - 0.80[d]
Tyrosine	0.21	0.21	0.19	0.29 - 0.47[c] 0.17 - 0.31[e]

a: Percentage on a dry weight basis
b: Unsprayed or sprayed with glufosinate-ammonium herbicide
c: (Watson 1982)
d: Data from analyses of 22 commercial Pioneer® Brand Hybrids
e: (Iowa Department of Agriculture and Land Stewardship 2001)

Table 19: Mean values of vitamin composition of grain (France and Italy, 1999)

Response variable[a]	1507 (unsprayed)[b]	1507 (sprayed)[b]	Non-GM control	Published range of values[c]
Thiamine hydrochloride (B1)	3.502	3.874	3.818	3.0 – 8.6
Riboflavin (B2)	1.208	1.199	1.314	0.25 – 5.6
Folic acid	0.158	0.161	0.154	0.3[d]
Total tocopherols	28.51	29.30	29.24	42 - 87

a: Parts per million (ppm) on a dry weight basis
b: Unsprayed or sprayed with glufosinate-ammonium herbicide
c: (Watson 1982)
d: (Watson 1987) reports an average value for folic acid in grain as 0.3 mg/kg

2.7b Other constituents including e.g. metabolites

In addition to a comparative analysis of the key constituents, comparative analyses should also be provided for any other constituents that may potentially be influenced by the genetic modification. For example, if the genetic modification may result in downstream metabolic effects.

Grain from maize line 1507 either sprayed or unpsrayed with glufosinate-ammonium, and the control maize was analysed for five secondary metabolites and two potential anti-nutrients (Table 20). There were no statistically significant differences between and control maize for the levels of inositol, p-coumaric acid and ferulic acid. The furfural concentration could not be determined as it was below the limit of quantitation (0.500 mg/100 g) of the assay. The levels of raffinose were within the range published in the literature and there were no significant differences between and control maize.

Phytic acid levels in grain from both unsprayed and sprayed and control maize were found to be within the published literature range for maize grain. Maize contains very low levels of the anti-nutrient trypsin inhibitor (Del Valle *et al.* 1983; Watson 1987). As expected, the trypsin inhibitor levels in both and control maize were below the limit of quantitation (2000 TIU/g) of the enzyme assay used in these analyses. This confirms that no unusually high levels of trypsin inhibitor were present in maize line 1507.

Table 20: Mean values of secondary metabolites and anti-nutrient composition of grain (France and Italy, 1999) Chile data

Secondary metabolites in grain				
Response variable[a]	**1507 (unsprayed)[b]**	**1507 (sprayed)[b]**	**Non-GM control**	**Published range of values[c]**
Inositol mg/100 g	45.75	48.303	45.43	NA
Raffinose %	0.099	0.089	0.107	0.08 – 0.30
p-Coumaric acid mg/100 g	20.34	20.98	16.83	NA
Furfural mg/100g	<LOQ[d]	<LOQ[d]	<LOQ[d]	NA
Ferulic Acid mg/100 g	273.85	284.51	260.54	NA
Anti-nutrient analyses of grain				
Phytic acid %	1.03	0.98	0.969	0.7 – 1.0
Trypsin inhibitor TIU/g	<LOQ[e]	<LOQ[e]	<LOQ[e]	NA

a: Percentage (%) or mg/100g on a dry weight basis. Trypsin inhibitor is expressed in units of trypsin inhibitor enzyme activity per gram on a dry weight basis
b: Unsprayed or sprayed with glufosinate-ammonium herbicide
c: (Watson 1982)
d: Below level of quantitation for furfural of 0.500 mg/100 g
e: Below level of quantitation for trypsin inhibitor of 2000 TIU/g
NA = ranges are not available in the published literature

2.7c Allergenic proteins

The levels of any naturally occurring allergenic proteins in the GM organism should be compared with the levels in the non-GM counterpart. Particular attention should be paid to the food groups considered most allergenic (peanuts, soybeans, milk, eggs, fish, crustacea, cereals and tree nuts) and where significant alterations to protein content are expected or could reasonably be anticipated;

Maize is not considered to have harmful toxicants and the genetic modification in maize line 1507 does not introduce any new toxicants harmful to humans, as discussed in 2.5e above. In addition, and as shown by the compositional analyses presented in 2.7 above, the levels of existing potential anti-nutrients in maize line 1507 are comparable to those of traditionally-bred (non-GM) maize.

2.8 Nutritional Impact

2.8a Animal feeding studies

Although not essential in all cases, animal-feeding studies may be provided as confirmation that the food is nutritionally adequate and that it will support typical growth and well being. Applicants are advised that such studies are not to be used as a substitute for careful molecular and comparative analyses of the food. Animal feeding studies are considered particularly useful where there may be changes in the bioavailability of certain nutrients or if the composition of the GM food is not comparable to any conventional foods.

Nutritional equivalence of maize line 1507 – poultry feeding study

Nutritional equivalence between maize line 1507 and traditionally-bred maize has also been shown in a poultry feeding study where broiler chickens were fed over a 42-day period with diets containing maize line 1507 grain or yellow dent grain from traditionally-bred maize. The mortality, body weight gain and feed conversion of the chickens fed with this maize were compared and the results are summarised in Table 21. No significant differences were observed on mortality, body weight gain or feed conversion between chickens fed a diet containing grain from maize line 1507 or grain from traditionally-bred maize (Zeph 2000).

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Table 21. Mortality, weight gain and feed conversion of chickens fed GM and non-GM maize.

Parameter	Treatment					
	Control (Bin #1)	Control (Bin #2)	Control (Bin #3)	Control (Bin #4)	Non-GM control hybrid	maize line 1507
Mortality (%)	5.71 [a]	5.71 [a]	2.86 [a]	5.71 [a]	2.86 [a]	5.71 [a]
Body Weight (kg) Day 0	0.044 [a]	0.043 [a]	0.043 [a]	0.043 [a]	0.044 [a]	0.043 [a]
Body Weight (kg) Day 42	1.730 [a]	1.739 [a]	1.738 [a]	1.728 [a]	1.739 [a]	1.757 [a]
Daily Gain (g per bird per day)	0.040 [a]	0.040 [a]	0.040 [a]	0.040 [a]	0.040 [a]	0.041 [a]
Feed Conversion (Body weight corrected)	1.797 [a]	1.806 [a]	1.808 [a]	1.804 [a]	1.802 [a]	1.775 [a]

a: Treatment means within a row with a common letter are not significantly different ($p < 0.05$)

2.8b Possible human nutritional impact

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

There have been no significant nutritional changes to maize line 1507 as a result of the modification. It is therefore concluded that there will be no impact on human nutrition as a result of substitution of maize products with maize line 1507.

2.9 Detection Methodology

Provide a description of the methodology that can be used to detect the genetic modification in any end products.

A PCR detection method to confirm molecular identity is being developed and a description of the draft methodology is provided (GeneScan GmbH 2001).

An ELISA Lateral Flow Test Kit is also under development to provide qualitative determination of the presence of the Cry1F delta endotoxin protein (Strategic Diagnostics Inc 2001).

These detection methods will be available prior to importation of corn products derived from maize line 1507 into Australia and New Zealand. It is also proposed that an independent third-party laboratory will be certified to use the methods provided by Dow AgroSciences to ensure the integrity, appropriate use and valid interpretation of test results.

The methods supplied in this submission are Confidential Business Information and should not be made available to any party or made available for public access.

Part 3 Regulatory/Legislative Details

3.1 Other Approvals

3.1a Overseas Regulatory Approvals

Provide information on any approvals that have been granted by overseas regulatory bodies that may be relevant to the proposed use of the food or food ingredient.

USEPA have approved maize line 1507 for commercial use in the USA until September 30, 2001. All existing B.t corn and cotton plant-pesticides are due for re-assessment by this date (EPA 2001). Approval was also granted by the U.S. Department of Agriculture (USDA) and the Food and Drug administration (FDA).

Submissions have been made to Canada, Argentina, the EU, Japan and Korea to permit import of maize products derived from maize line 1507.

3.1b Regulatory disapproval

Provide information on whether approval has been rejected or withdrawn by any regulatory body.

There have been no rejections or withdrawals of submissions for food safety assessment of maize line 1507.

3.2 Regulatory Impact Statement

Please identify the economic implications associated with your application. Relevant quantitative and qualitative information the Authority needs could include:

- *cost implications*
- *profit implications*
- *market share implications*
- *price implications*
- *trade implications*
- *employment implications*

This application to the Australian and New Zealand Food Authority has been largely based on the submission generated for other overseas agencies and for the US approval process to receive approval to cultivate maize line 1507. It is a necessary component of the Dow AgroSciences global approval process since without such food import approvals, the cultivation and marketing of maize line 1507 in the USA will be significantly hampered. The profit and market share implication are difficult to quantify, however, freedom to operate in the marketplace is a significant market advantage and will have an impact on both factors.

The local cost implications are made up of personnel time both locally and globally as well as the direct fees associated with the submission. The personnel costs are estimated to be approximately US$18,000 (15 days at full economic cost of US$1200 per day). The submission fees are expected to be significant.

There are few price or employment implications which are directly related to the ANZ assessment of maize line 1507. The product has been launched in the USA although full commercial releases are not planned until 2002.

The trade implications are reasonably clear since non-approval by ANZFA would impose a trade restriction on maize line 1507 and products derived from these lines.

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