Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268-1054

00-136-01p
CBI-deleted

308/3E
May 10, 2000

Dr. John Payne
Assistant Director
Scientific Services
USDA, APHIS, PPQ, Unit 133
4700 River Road
Riverdale, MD 20737-1237
(301) 734-8896

Dow AgroSciences™

PETITION FOR DETERMINATION OF NONREGULATED STATUS
B.T. CRY1F INSECT RESISTANT, GLUFOSINATE TOLERANT MAIZE LINE

Dear Dr. Payne:

Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International, Inc. are submitting a petition to request a determination from the Animal and Plant Health Inspection Service (APHIS) that maize transformation event TC1507 and any maize lines derived from crosses with *B.t.* Cry1F maize line 1507 no longer be considered regulated articles under 7 CFR Part 340.

Enclosed is a petition for a determination on the regulatory status of *Zea mays* L. cultivar "Line 1507," which has been modified to be resistant to feeding from certain lepidopteran species and resistant to the herbicide glufosinate, and is currently deemed a "regulated article". Based on the data and information contained in the enclosed petition, we believe that there is no longer "reason to believe" that the modified maize plant should be considered to be a regulated article. The modified maize plant does not present a plant pest risk and is not otherwise deleterious to the environment.

Contents of Submission:

- CBI version of Petition (Petition plus 24 additional CBI volumes)
  (two original documents)
- CBI deleted version of Petition
  (two original documents plus four additional copies)

The undersigned certifies that, to the best of her knowledge and belief, this petition includes all data, information, and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is the subject of the petition.

If you require further information, please contact me at (858) 352-4438 or Renee Bartz, Registration Assistant for this product at (317) 337-4673.

Sincerely,

Diane Shanahan

Diane M. Shanahan
Regulatory Manager
Regulatory Success - Americas

DMS/rb
Enclosures

5/15/2000

00-136-01p
CBI-deleted

PETITION FOR DETERMINATION OF NON-REGULATED STATUS
B.t. Cry1F INSECT-RESISTANT, GLUFOSINATE-TOLERANT MAIZE LINE 1507

CBI DELETED COPY

The undersigned submits this petition under 7 CFR 340.6 to request that the Administrator, Animal and Plant Health Inspection Service (APHIS), make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by

Diane Shanahan
Regulatory Manager

Mycogen Seeds c/o Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
USA

Telephone: 858-352-4438
Fax: 858-352-4466

Cynthia Stauffer
Regulatory Affairs Manager

Pioneer Hi-Bred Intl. Inc.
P.O. Box 552
Johnston, IA 50131-1000

Telephone: 515-270-3995
Fax: 515-334-4478

Contributors:

L. Zeph and T. Rood, Pioneer Hi-Bred Intl. Inc.
R. Sibley, Mycogen Seeds c/o Dow AgroSciences LLC

May 10, 2000

5/15/2000

Summary

Mycogen Seeds c/o Dow AgroSciences LLC (Mycogen) and Pioneer Hi-Bred International, Inc. (Pioneer) are submitting a Petition for Determination of Nonregulated Status for *B.t.* Cry1F insect-resistant, glufosinate-tolerant maize line 1507. Mycogen and Pioneer request a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that maize transformation event TC1507, and any maize lines derived from crosses with *B.t.* Cry1F maize line 1507, no longer be considered regulated articles under 7 CFR Part 340.

B.t. Cry1F maize line 1507 is considered a regulated article because the transformation event contains promoter and terminator sequences from the plant pests *Agrobacterium tumefaciens* and cauliflower mosaic virus. Small-scale field tests have shown that *Bacillus thuringiensis* var. *aizawai* Cry1F insect control protein as expressed in maize is very effective in controlling certain lepidopteran larva that are common pests of maize, such as European corn borer (ECB) *Ostrinia nubilalis* (Hubner), southwestern corn borer *Diatraea grandiosella*, black cutworm *Agrotis ipsilon*, and fall armyworm *Spodoptera sp.*

The original TC1507 transformation event was produced by Pioneer using Mycogen's plant optimized (po) *cry*1F truncated gene derived from the *Bacillus thuringiensis* var. *aizawai* strain PS811. *Bacillus thuringiensis*, a common Gram-positive soil bacterium, produces an insecticidal protein that is very selective in toxicity to specific organisms. Decades of safety testing on *B.t.* protein demonstrate the lack of toxicity to humans and animals, and the absence of adverse effects on non-target organisms and the environment (EPA, 1998).

The *pat* gene, which encodes the enzyme phosphinothricin acetyltransferase (PAT), is also present in *B.t.* Cry1F maize line 1507. The *pat* gene is a synthetic version based on the native *pat* gene from *Streptomyces viridochromogenes*, a non-pathogenic bacterium. The inclusion of the *pat* gene enables plant selection of the *B.t.* lines and provides tolerance to glufosinate-ammonium herbicides. The PAT protein does not confer pesticidal activity, however, it does provide an alternative weed management tool to growers. *B.t.* Cry1F maize line 1507 may 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 and there are no known adverse environmental or toxicological effects.

B.t. Cry1F maize line 1507 has been field tested since 1997 in the major maize growing regions of the United States as well as in Puerto Rico and Hawaii. All field tests have occurred under field notifications granted by USDA - APHIS. Information collected during those trials indicates that *B.t.* Cry1F maize line 1507 exhibits no plant pathogenic properties and is unlikely to harm other insects that are beneficial to agriculture. *B.t.* Cry1F maize line 1507 is no more likely to be a weed than non-transgenic maize and expression of the Cry1F protein is unlikely to increase the weediness potential of any other cultivated plant or wild species. In summary, *B.t.* Cry1F maize line 1507 is not likely to:

- become a weed of agriculture or be invasive of natural habitats,
- cross with wild relatives and create hybrid offspring which may become weedy or invasive,
- become a plant pest,
- have an impact on non-target species, including humans,
- have an impact on biodiversity.

5/15/2000
1Cay

Statement of Grounds Unfavorable

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

Diane Shanahan
Regulatory Manager

Mycogen Seeds c/o Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
USA

Telephone: 858-352-4438
Fax: 858-352-4466

Cynthia Stauffer
Regulatory Affairs Manager

Pioneer Hi-Bred Intl. Inc.
P.O. Box 552
Johnston, IA 50131-1000

Telephone: 515-270-3995
Fax: 515-334-4478

PETITION FOR DETERMINATION OF NON-REGULATED STATUS OF *B.t.* CRY1F INSECT-RESISTANT GLUFOSINATE-TOLERANT MAIZE LINE 1507

Table of Contents

CBI Deleted Copy

Tables

Figures

Attachments

Appendices

CBI Deleted Copy

Volume No.	Title	Author	No. of Pages
Volume 1 **CBI Deleted**	[Microbial B.t. Cry1F (truncated) Delta-Endotoxin: Maize-Insect-Pest Susceptibility Study]	Herman, R.A. and Korjagin, V.A.	22
Volume 2 **CBI Deleted**	[Characterization of Inserted Genes in Cry1F Maize Line 1507]	Hondred, D.	17
Volume 3 **CBI Deleted**	[Phosphinothricin acetyltransferase (PAT) protein: In Vitro Digestibility Study]	Glatt, C.M.	17
Volume 4 **CBI Deleted**	[Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures]	Young, D.L. and Herman, R.A.	71
Volume 5 **CBI Deleted**	[Non-target Exposure and Risk Assessment for Environmental Dispersal of Cry1F Maize Pollen]	Wolt, J.D.	244
Volume 6 **CBI Deleted**	[Environmental Fate of Cry1F Protein Incorporated into Soil]	Halliday, W.R.	87
Volume 7 **CBI Deleted**	[Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate]	Hoxter, K., Porch, J., Krueger, H.	32
Volume 8 **CBI Deleted**	[Chronic Exposure of Folsomia Candida to Bacterially Expressed Cry1F Protein]	Halliday, W.R.	122
Volume 9 **CBI Deleted**	[B.t. Cry1F Delta-Endotoxin: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (Daphnia magna) Using Bacterially Expressed B.t .Cry1F Delta-Endotoxin, and Pollen from Maize Expressing B.t. Cry1F Delta-Endotoxin]	Drottar, K.R. and Krueger, H.O.	21
Volume 10 **CBI Deleted**	[Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed B.t. Cry1F Delta-Endotoxin and Pollen from Maize Expressing B.t. Cry1F Delta Endotoxin]	Maggi, V.L.	53
Volume 11 **CBI Deleted**	[Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: A Dietary Toxicity Study with Green Lacewing Larvae]	Hoxter, K., Porch, J., Krueger, H.	28

Volume No.	Title	Author	No. of Pages
Volume 12 **CBI Deleted**	[Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: A Dietary Toxicity Study with the Ladybird Beetle]	Hoxter, K., Porch, J., Krueger, H.	32
Volume 13 **CBI Deleted**	[Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera]	Hoxter, K., Porch, J., Krueger, H.	33
Volume 14 **CBI Deleted**	[Waiver Request: Fish Toxicity Text With Transgenic Maize (Corn) Containing Bacillus thuringiensis var. aizaqoi (B.t.) Cry1F Delta-Endotoxin]	Mayes, M.	18
Volume 15 **CBI Deleted**	[Transgenic Corn Expressing Bacillus thuringiensis var. aizawai (B.t.) Cry1f Delta Endotoxin: A Dietary Toxicity Study with the Northern Bobwhite]	Gallagher, S., Grimes, J., Beavers, J.	38
Volume 16 **CBI Deleted**	[Field Survey of Beneficial Arthropods Associated with Bacillus thuringiensis Cry1F Maize]	Higgins, L.	20
Volume 17 **CBI Deleted**	[Efficacy of Cry1F Events TC1360 and TC1507]	Moellenbeck, D. and Peters, M.	89
Volume 18 **CBI Deleted**	[Cry1F Binding Studies]	Masson, L., Adang, M. and Schwab, G.	38
Volume 19 **CBI Deleted**	[Resistance Management Plan for Transgenic Maize Expressing the Cry1F Insecticidal Protein from Bacillus thuringiensis var. aizawai]	Bystrak, P. and Moellenbeck, D.	20
Volume 20 **CBI Deleted**	[Supplement to MRID 44714801: Supplemental Data - Product Characterization Data for Bacillus thuringiensis var. aizawai Cry1F Insect Control Protein as Expressed in Maize]	Shanahan, D.	78
Volume 21 **CBI Deleted**	[Supplement to MRID 44691101: Supplemental Data for Acute Oral Toxicity Study in Mice: Cry1F Bacillus thuringiensis var. aizawai delta-endotoxin]	Shanahan, D.	17
Volume 22 **CBI Deleted**	[Acute Oral Toxicity Study in Mice]	Kuhn, J.	11
Volume 23 **CBI Deleted**	[Comparison of Amino Acid Sequence Similarity of Cry1F and PAT Proteins to Known allergen Proteins]	Meyer, T.	24
Volume 24 **CBI Deleted**	[Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterization of Text Substances for Biochemical and Toxicological Studies]	Evans, S.	298

Abbreviations and Scientific Terms

A+T	Adenosine and Tyrosine
APHIS	Animal and Plant Health Inspection Service
Bla	β lactamase gene
B.t.	*Bacillus thuringiensis*
BCW	Black Cutworm, *Agrotis ipsilon*
CEW	Corn Earworm, *Heliothis zea*
CaMV	Cauliflower Mosaic Virus
Cry1Ab	Insecticidal crystal protein from *Bacillus thuringiensis* var. *kurstaki*
Cry1F	Insecticidal crystal protein from *Bacillus thuringiensis* var. *aizawai*
*cry*1F	Synthetic gene that encodes a truncated Cry1F insecticidal protein
*cry*1Fa2	Native gene that encodes a full length Cry1F insecticidal protein
DNA	Deoxyribonucleic Acid
ECB	European corn borer, *Ostrinia nubilalis* (Hubner)
ELISA	Enzyme Linked Immunosorbent Assay
EPA	U.S. Environmental Protection Agency
FAW	Fall Armyworm, *Spodoptera frugiperda*
G+C	Guanine and cytosine nucleotides
ICP	Insecticidal crystalline protein
kb	Kilo-base pairs
kDa	Kilo Dalton, a measurement of protein molecular weight
mRNA	Messenger ribonucleic acid
*Nhe*I	Restriction enzyme site
ORF25PolyA	Terminator from *Agrobacterium tumefaciens* pTi15955
PAT	Phosphinothricin acetyltransferase enzyme
(po) *cry*1F	Plant optimized *cry*1F gene
pat	Phosphinothricin acetyltransferase gene
PHI8999A	Linear portion of DNA containing *cry*1Fa2 and *pat* coding sequences
PHP8999	Plasmid DNA containing *cry*1Fa2 and *pat* coding sequences
PCR	Polymerase Chain Reaction
*Pme*I	Restriction enzyme site
RNA	Ribonucleic acid
*Sph*I	Restriction enzyme site
*Stu*I	Restriction enzyme site
SWCB	Southwestern corn borer, *Diatraea grandiosella*
TBW	Tobacco budworm, *Heliothis virescens*
TC1507	Transformation event that encodes an insecticidal protein and confers herbicidal tolerance
Trunc	Truncated
UBIZM1(2)	*Zea mays* ubiquitin promoter plus ubiquitin intron and a 5' untranslated region
USDA	United States Department of Agriculture
V4 to V9	Maize plant vegetative stage 4 to 9
*Xho*I	Restriction enzyme site
35S	Promoter and terminator from cauliflower mosaic virus

I. Rationale

The commercial introduction of transgenic maize expressing the *B.t.* toxin has, for the first time, provided growers with a simple, cheap, highly effective, and environmentally benign means of controlling the European corn borer (ECB) [*Ostrinia nubilalis (Hubner)*]. Damage by the European corn borer currently costs growers 5-20% yield loss. When chemical insecticides are used, only marginal efficacy is obtained due to the tissue boring nature of the pest. Mycogen and Pioneer have developed *B.t.* Cry1F maize line 1507, containing transformation event TC1507, which expresses the Cry1F protein from the bacterium *Bacillus thuringiensis* var. *aizawai*. The transformation event TC1507 was created when insert DNA (PHI8999A) was incorporated into maize callus tissue. When the tissue was regenerated into a maize plant, the resulting plant was referred to as *B.t.* Cry1F maize line 1507. The progeny derived from *B.t.* Cry1F maize line 1507 will be the first to use an insect control protein derived from *Bacillus thuringiensis* var. *aizawai*. As such, *B.t.* Cry1F maize line 1507 demonstrates a broader spectrum of activity and represents an excellent addition to growers' options for insect control in maize. *Bacillus thuringiensis* var. *aizawai* Cry1F insect control protein as expressed in maize is very effective in controlling certain Lepidopteran larva that are common pests of maize, such as European corn borer (ECB) *Ostrinia nubilalis* (Hubner), southwestern corn borer *Diatraea grandiosella*, black cutworm *Agrotis ipsilon* and fall armyworm *Spodoptera sp.*

The *pat* gene, which encodes the enzyme phosphinothricin acetyltransferase (PAT), is also present in *B.t.* Cry1F maize line 1507. The *pat* gene is a synthetic version based on the native *pat* gene from *Streptomyces viridochromogenes*, a non-pathogenic bacterium. The inclusion of the *pat* gene enables plant selection of the *B.t.* lines and provides tolerance to glufosinate-ammonium herbicides. The PAT protein does not confer pesticidal activity, however, it does provide an alternative weed management tool to growers. *B.t.* Cry1F maize line 1507 may 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 and there are no known adverse environmental or toxicological effects.

II. The Maize Family (*Zea mays* L.)

II.A. Maize as a crop

Maize has been cultivated in North America for thousands of years (Canadian Food Inspection Agency, 1994). Maize grain has been produced primarily from hybrid-derived varieties since the 1930's and 1940's. Hybrid varieties are superior to open-pollinated varieties in a variety of agronomic characteristics, for example, in grain yield.

The primary use of maize is for animal feed; but maize is also processed into valuable food and industrial products, such as ethyl alcohol by fermentation, cornmeal by dry milling, and highly refined starch by the wet milling process. The yellow dent type of maize is used for feed and is grown as a commodity crop in North America. Wet milling to produce starch and sweetener products for foods accounts for the greatest volume of maize that is processed. Non-food products such as industrial starches, corn gluten feed, and corn gluten meal are also manufactured (White and Pollak, 1995). The primary products derived from the dry milling process are corn grits, cornmeals, and corn flours. The largest food/feed product volume of the dry-milling industry is animal feed followed by brewing and food uses.

II.B. Taxonomy of maize

Family name:	Gramineae
Genus:	*Zea*
Species:	*mays* (2n = 20)
Subspecies:	none

A summary description of the taxonomy of maize can be found in Canadian Food Inspection Agency (1994). *Zea mays* is a member of the Gramineae (Poaceae) family, commonly known as the grass family. Maize is a monoecious annual species with separate staminate (tassels) and pistillate (silk) flowers. The plant architecture is characterized by overlapping sheaths and broad, conspicuously distichous blades with numerous staminate spikelets in long spikelike racemes, forming large spreading terminal panicles (tassels). Pistillate (silk) inflorescence is in the axis of the leaves on a thickened, almost woody axis (cob). The silks are composed of numerous large foliaceous bracts or spathes, with long styles protruding from the summit as a mass of silky threads.

II.C. Genetics of maize

The occurrence of separate staminate and pistillate flowers encourages the natural outcrossing between maize plants (Canadian Food Inspection Agency, 1994). Hybridization is achieved through repeated cycles of self-pollination leading to homogeneity of the genetic characteristics within a single plant line (inbred). Controlled cross-pollination of inbred lines from chosen genetic pools combines desired genetic traits in a hybrid and results in a yield increase. This inbred-hybrid concept, and the resulting yield response, is the basis of the modern maize seed industry. Maize varieties planted by North American farmers are almost entirely hybrids.

II.D. Pollination of maize

Maize is a wind-pollinated plant. The separate tassels and silk flowers encourage the natural outcrossing between maize plants. Typical of wind-pollinated plants, a large amount of redundant maize pollen is produced for each successful fertilization of an ovule on the ear (Kiesselbach, 1949). Wind movements across the maize field cause pollen from the tassel to fall on the silks of the same or adjoining plants. Maize pollen measures about 0.1 mm in diameter and is the largest of any pollen normally disseminated by wind from a comparably low level of elevation. Dispersal of maize pollen is influenced by its comparatively large size and rapid settling rate with less then 1% of field pollen transporting beyond 60 meters from the field edge (Raynor *et al.*, 1972). Pollen survival is highly dependent on temperature and relative humidity. Maize pollen remains viable for about 30 minutes under optimal conditions of temperature and humidity (Canadian Food Inspection Agency, 1994).

II.E. Weediness of maize

Maize does not exhibit any weedy tendencies and is non-invasive in natural ecosystems (Canadian Food Inspection Agency, 1994). Maize hybrids have been domesticated for such a long period of time that the seeds cannot be separated from the cob and disseminated without human intervention. Maize plants are annuals that will not survive in the U.S. from one growing season to the next because of the poor dormancy. Maize seed is non-dormant, but can persist from one growing season to the next under favorable climatic conditions and, when the temperature and moisture are adequate the seed will germinate. These volunteers are easily identified and controlled through manual or chemical means. Some *Zea* species are successful wild plants in Central America, but they have no pronounced weedy tendencies.

II.F. Modes of introgression in maize

Cultivated maize (*Zea mays* ssp. *mays*) belongs to the genus *Zea*, which includes several other wild species, collectively known as teosintes. The closest species related to maize is teosinte (*Zea mexicana*), a wild grass found in Mexico and Guatemala. No sexually compatible wild relatives of maize are found in the U.S. and Canada. A study examining genetic variability by allozyme analysis of maize and teosinte gene bank accessions failed to show any evidence of recent introgression between maize and teosinte (Smith *et al.*, 1985). *Z. mays* does not appear to be a hybrid of the wild and cultivated forms of *Zea* as had been previously postulated. Therefore, there is no readily available genetic bridge between cultivated *Z. mays* and wild *Zea* (Doebley, 1984).

The genus most closely related to *Zea* is *Tripsicum*, a genus of seven species. Three species of *Tripsicum* occur in the U.S. Only one species, *Tripsacum dactyloides* (Eastern Gamagrass) is found in the maize growing regions of the Midwestern U.S. Crosses can be made between *Z. mays* and *T. dactyloides*, but these require human intervention and progeny are frequently sterile or genetically unstable (Manglesdorf, 1974).

II.G. Characteristics of non-transformed cultivar

The maize germplasm utilized as the initial recipient of the added genes is a public inbred line designated Hi-II.

III. Description of the Transformation System

For transformation of maize tissue a linear portion of DNA, containing the *cry*1F gene and the *pat* selectable marker gene, was excised from a complete plasmid. This linear portion of DNA, termed an insert, was used in the transformation process. A complete description of plasmid PHP8999 (9504 bp), from which the insert was derived, is shown in Table 1, and a plasmid map of PHP8999 is presented in Figure 1. *Pme*I digestion of PHP8999 provided the 6.2 kb insert, designated PHI8999A, that was used in transformation (Fig. 2).

B.t. Cry1F maize plants were obtained by microprojectile bombardment using the Biolistics® PDS-1000He particle gun manufactured by Bio-Rad, essentially as described by Klein *et al.* (1987). Immature embryos isolated from 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 purified PHI8999A DNA and accelerated into the cultured embryos, where the insert DNA was incorporated into the cell chromosome. Only insert PHI8999A was used during transformation and no additional plasmid DNA was incorporated into the transformant. After bombardment, embryos were transferred to callus initiation medium containing glufosinate as the selection agent. 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-resistant callus tissue were assigned unique identification codes representing putative transformation events, and continually transferred to fresh selection medium. 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 transgenes 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. Line 1507 was selected for its good agronomic characteristics and excellent resistance to European corn borer and other insects.

Table 1. Genetic elements of plasmid PHP8999

Genetic element	Size (kb)	Function
*ubi*ZM 1(2)	1.98	The ubiquitin promoter (plus ubiquitin intron and a 5' untranslated region) from *Zea mays* (Christensen *et al.*, 1992)
*cry*1F (trunc)	1.82	A synthetic version of truncated *cry*1F from *Bacillus thuringiensis* var. *aizawai* (plant optimized)
ORF25PolyA	0.72	A terminator from *Agrobacterium tumefaciens* pTi15955
CaMV 35S promoter	0.55	35S promoter from Cauliflower Mosaic Virus (Odell *et al.*, 1985)
pat	0.55	The synthetic glufosinate resistance gene (plant optimized), based on a phosphinothricin acetyltransferase gene sequence from *Streptomyces viridochromogenes* (Eckes *et al.*, 1989)
CaMV 35S terminator	0.20	35S terminator from Cauliflower Mosaic Virus
nptII	0.81	Neomycin phosphotransferase type II gene that confers bacterial kanamycin resistance; driven by bacterial promoters that do not contain the regulatory elements necessary to express in plants..
ori-pUC19	2.87	The origin of replication from backbone plasmid pUC19 derived from E. coli pMB1 (ColE1 and pBR322). (Yanisch-Perron, *et al.*,. 1985).

Figure 1. Plasmid PHP 8999

Pertinent restriction sites and genetic elements are indicated. Event TC1507 was generated using the 6235 bp DNA fragment created by digestion with the restriction endonuclease *Pme* I.

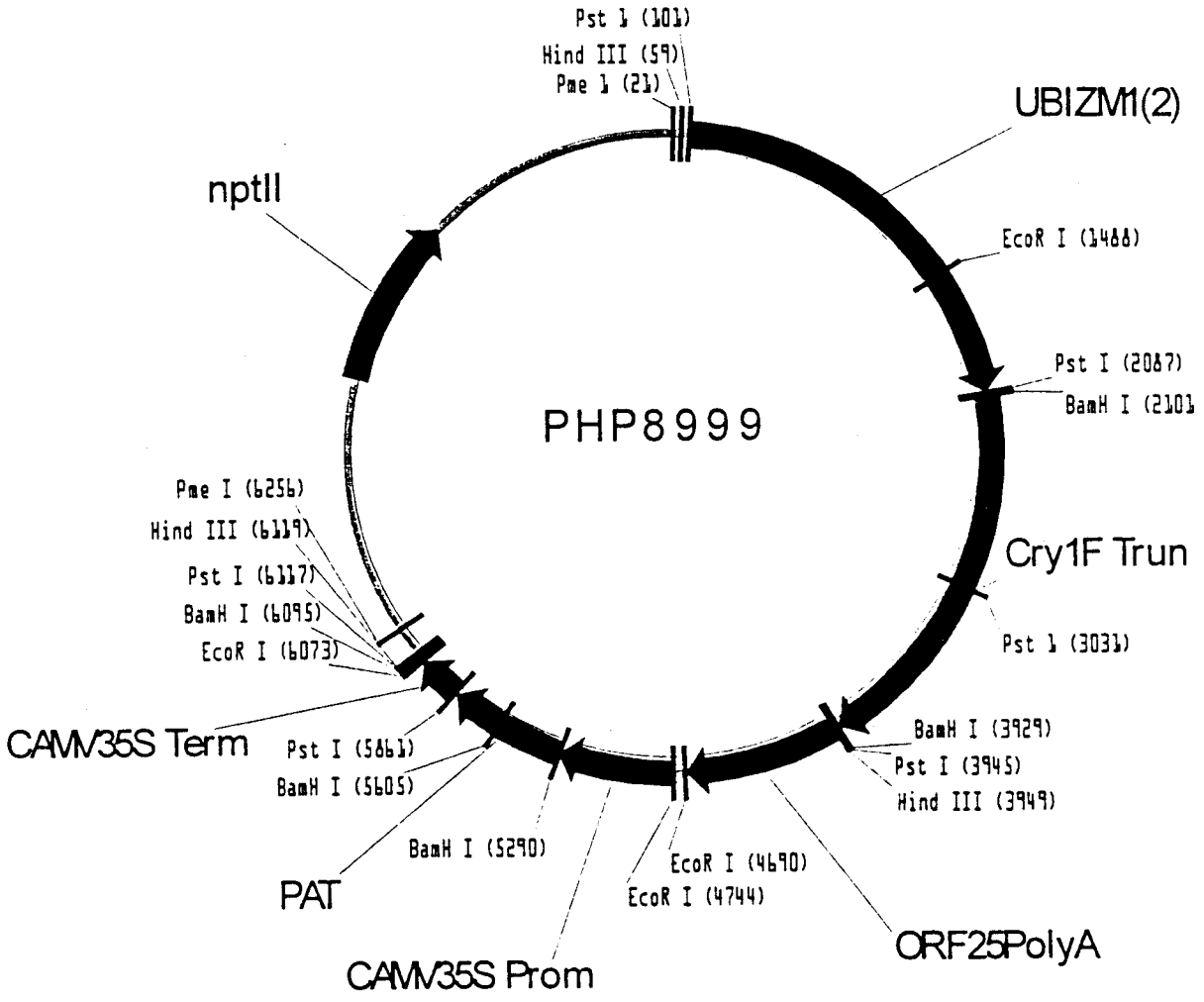
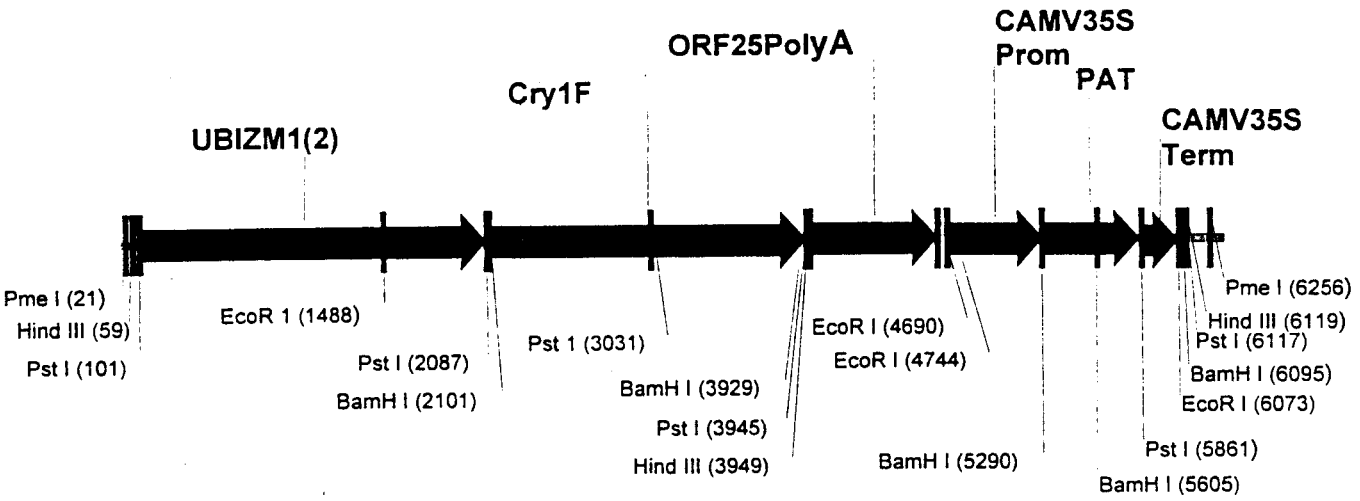


Figure 2. Insert PHI 8999A



IV. The Donor Genes and Regulatory Sequences

IV.A. The *cry*1F gene

The insecticidal protein that is the subject of this petition is a truncated Cry1F protein derived from *Bacillus thuringiensis* var. *aizawai* strain PS81I (NRRL B-18484). A synthetic *cry*1F transgene optimized for plant codon usage was transformed into maize plants. This resulted in Cry1F expression levels that were efficacious for control of the lepidopteran pest European corn borer in transgenic maize lines. The insecticidal protein encoded by the synthetic *cry*1F transgene is identical in sequence to amino acids 1-605 of the native, 1174 amino acid residue Cry1F protein except for a single amino acid residue substitution (see below). The codons for the C-terminal 569 amino acids of the full-length protoxin correspond to those removed by alkaline proteases in the insect gut during formation of the active Cry1F toxin. These amino acid residues were not included in the design of the transgene sequence. A complete description of the *cry*1F transgene construct, including expression elements and the selectable marker, can be found in Table 1 of this submission.

Brief review of *Bacillus thuringiensis* δ-endotoxins and transgene design.

Bacillus thuringiensis is a diverse group of Gram-positive, spore-forming bacteria best known as a valuable source of commercially important biopesticides. A well-studied class of *B.t.* insecticidal proteins is the δ-endotoxins. Advances in agricultural biotechnology have enabled the expression of *B.t.* toxins in transgenic plants, thereby imparting intrinsic insect resistance traits to a number of important crops (Prieto-Samsonov *et al.*, 1997).

Many natural δ-endotoxins are produced as insoluble parasporal crystalline inclusions 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 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 microvillae of epithelial midgut cells. Binding of the activated toxin is followed by a conformational change of the toxin and its insertion into the membrane. Toxin oligomerization then results in the formation of pores in the cell membrane of the midgut cells and osmotic cell lysis leading to insect death.

The amino-terminal, protease-resistant core of Cry1 δ-endotoxins is comprised of three structural domains (Grochulski *et al.*, 1995). Domain 1, the pore-forming domain, consists of a bundle of seven anti-parallel α-helices in which helix-5 is encircled by the remaining helices. Domain 2 consists of three anti-parallel β-sheets joined in a "Greek-key" topology. Loops extending from domain 2 are involved in receptor binding and insect cross-resistance correlates with domain 2 sequence similarity (Tabashnik *et al.* 1996). Domain 3 is a β-sandwich of two antiparallel β-sheets. This domain is proposed to stabilize the toxin. In addition, recent evidence demonstrates involvement of domain 3 in receptor binding (de Maagd *et al.* 1996; Lee *et al.* 1995; Bosch *et al.* 1994).

Expression of *B.t.* δ-endotoxin genes in plants was first reported in the scientific literature using native bacterial genes encoding the entire protoxin (Adang *et al.*, 1987). In such instances, *B.t.* δ-endotoxin expression at the RNA transcript or protein level was undetectable or very low; however, insect toxicity was observed. Expression of native *B.t.* genes was measurable at the RNA or protein level when the genes were truncated to encode only the protease-resistant core toxin comprised of the three domains described above (Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987). Examination

of mRNA in plants transformed with native gene sequences has revealed the presence of many species shorter than the expected full-length size, suggesting improper postranscriptional processing or rapid mRNA turnover (Barton *et al.*, 1987; Adang *et al.*, 1987). Evidence favoring the latter mechanism was provided in a study by Murray *et al.* (1991).

Since these early studies, numerous reports have demonstrated that more efficient expression of *B.t.* toxins in plants can be achieved using synthetic transgenes in which the relatively low G+C content of native bacterial δ-endotoxin genes is altered to more closely approximate the higher G+C content typically found in plant genes. In designing the *cry*1F gene, substitutions of alternative codons were used to bias the codon usage to that preferred by the target plant, and to remove certain deleterious A+T rich sequence elements responsible for mRNA destablization such as RNA cleavage signals, inappropriate polyadenylation sequences, intron splice sites, hairpin sequences, and transcription termination signals. The truncated, plant optimized *cry*1F was optimized in this manner using plant preferred codon usage tables (Murray *et al.*, 1989).

The promoter for the *cry*1F gene is the maize ubiquitin ZM1 promoter (*ubi*ZM1(2)) containing sequence from the first ubiquitin exon (Exon 1) and intron (Intron 1). This exon and intron pair appear to improve the expression level of coding regions with which they are associated. The start of transcription for the *ubi*ZM1 gene has been mapped to the beginning of Exon 1 (Christensen *et al.*, 1992). This report also further delineates the Exon 1, Intron 1, and Exon 2 regions of *ubi*ZM1. Inspection of the sequence for these regions indicates that the resulting mature, fully spliced mRNA has no ATG to initiate translation in Exon 1, and the first such ATG occurs in Exon 2. In the PHI8999 plasmid construct, the Ubi1 Exon 2 is replaced by a translation consensus sequence and an ATG for the start of translation of the Cry1F protein coding sequence. Like many 5'-proximal exons in plants and other eucaryotes the *ubi*ZM1 Exon 1 represents 5'-untranslated mRNA, which is not translated into any portion of the Cry1F protein.

The ORF25 polyA terminator consists solely of a terminator region from an open reading frame isolated from *Agrobacterium tumefaciens* strain pTi15955. The noncoding ORF25 polyA terminator does not cause the maize line to become a plant pest.

Peptide sequence of transgenic Cry1F.

The plant-optimized *cry*1F transgene encodes a truncated Cry1F protein that is identical to the active core toxin of both the native Cry1F full length protein from *Bacillus thuringiensis* var. *aizawai* and the microbially derived Cry1F full length protein, MR872. All three Cry1F proteins are identical for the first 605 amino acids, with the exception of the substitution of leucine at position 604 in the plant Cry1F protein ($F_{604}L$ substitution in Figure 3). This change in the coding sequence was made to introduce an *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.

MR872 is the strain designation of the *Pseudomonas fluorescens* chimeric Cry1F/Cry1Ab strain used for microbial production of the truncated Cry1F test material for toxicology. The sequences coding for the Cry1Ab C-terminal domain were fused with the Cry1F core toxin to enable high level expression of the full-length Cry1F protein in heterologous microbial host strains. After fermentation the full-length microbial Cry1F protein was truncated and prepared for use in toxicological studies and for use in the protein equivalency study which compares Cry1F protein from microbial and plant sources.

Figure 3. Alignment of the amino acid sequences of the delta-endotoxin proteins comparing a microbially-derived Cry1F protein (MR872), the truncated plant Cry1F protein (Cry1F syn), and *B.t.a* Cry1F full length protein (Cry1F).

The consensus sequence represents identical residues among all three genetic versions encoding the Cry1F protein. 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 a ↓. Note the single $F_{604}L$ substitution in the transgenic polypeptide. This difference is due to codon changes resulting from the introduction of a restriction enzyme site that enables gene cloning of the alternative C-terminal half of the protoxin. The consensus sequence represents identical residues among all three genetic versions encoding the Cry1F protein.

MR872 = Amino acid sequence of microbially-derived Cry1F protein used in toxicological studies.
Cry1Fsyn = Amino acid sequence of truncated plant Cry1F protein.
Cry1F = Amino acid sequence of native Cry1F protein from *Bacillus thuringiensis* var. *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

           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  ---------- ---------- -----
```

IV.B. The gene for resistance to glufosinate: phosphinothricin acetyltransferase

Brief review of the PAT protein and transgene design.

The PAT protein acetylates phosphinothricin, conferring tolerance to a chemically synthesized phosphinothricin such as the herbicide glufosinate-ammonium. Acetylation converts phosphinothricin to an inactive form that is no longer toxic to maize plants. Glufosinate ammonium is a broad spectrum, non-systemic, non-selective herbicide. Individual maize plants tolerant to glufosinate ammonium herbicide can be readily identified in the field through either spray application or application of the herbicide to leaves.

Expression of the PAT protein confers tolerance to application of glufosinate ammonium herbicide for purposes of weed control management. The PAT enzyme catalyzes the conversion of L-phosphinothricin, the active ingredient in glufosinate ammonium, to an inactive form and thereby detoxifies the glufosinate ammonium herbicide.

The *pat* gene is a synthetic version of the native bacterial *pat* gene sequence from *Streptomyces viridochromogenes* (Eckes *et al.*, 1989). The synthetic version was produced in order to modify the guanine and cytosine codon bias to a level more typical for plant DNA (Van Wert, 1994) as discussed in the product characterization section for the active ingredient, Cry1F. The synthetic, plant-optimized *pat* gene shows improved expression of PAT protein in maize plants.

The promoter and terminator sequences for the *pat* gene are from the cauliflower mosaic virus. CaMV is a double stranded DNA caulimovirus with a host range restricted to primarily cruciferous plants. CaMV 35S is a constitutive promoter and it is expected that this promoter will express proteins in most tissues of the whole plant. This was confirmed for the PAT protein by ELISA tests described in Section V. The CaMV sequences do not cause the maize line to become a plant pest.

Peptide sequence of transgenic PAT.

The plant-optimized *pat* transgene encodes a protein of 183 amino acids (Figure 4). The amino acid sequence of the PAT protein is identical to the PAT protein present in commercial maize hybrids with tolerance to glufosinate ammonium (Van Wert, 1994).

Figure 4. Amino Acid Sequence of the PAT Protein.

```
1                                                          50
MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
51                                                         100
ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101                                                        150
GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151                                 184
AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI*
```

V. Genetic Analysis and Agronomic Performance

The analysis demonstrates that event TC1507 contains: (1) the synthetic version of the truncated *cry1F* gene from *Bacillus thuringiensis* var. *aizawai* whose transcription is directed by the ubiquitin promoter (plus ubiquitin intron and a 5' untranslated region) from *Zea mays* and whose termination sequences were derived from, ORF25PolyA, a terminator from *Agrobacterium tumefaciens* pTi15995; (2) the synthetic glufosinate resistance gene (plant optimized), based on a phosphinothricin acetyltransferase gene sequence from *Streptomyces viridochromogenes* whose transcription is directed by CaMV 35S promoter from cauliflower mosaic virus and whose termination sequences were derived from CaMV 35S, a terminator from cauliflower mosaic virus. Both proteins are expressed in the plants containing event TC1507. The results of the detailed molecular characterization of event TC1507 support the conclusion that this event contains a full-length copy of the DNA insert used to transform this maize line (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.

The characterization and genetic stability of the inserted DNA in *B.t.* Cry1F maize line 1507 was confirmed by Southern analysis of DNA isolated from two generations in the breeding process for this line. The details of these analyses are summarized in sections V.A. and V.B, along with data on Mendelian segregation for *B.t.* Cry1F maize line 1507 in V.C. Both the *cry*1F and *pat* genes are expressed in *B.t.* Cry1F maize line 1507, conferring resistance to certain lepidopteran insect pests and tolerance to commercial levels of glufosinate herbicide. The details on expression of the Cry1F and PAT proteins can be found in sections V.D. and V.E. Details on the agronomic characteristics of *B.t.* Cry1F maize line 1507 can be found in section V.F. and V.G.

V.A. Characterization of the DNA insert

Experimental design and methods

Seeds were obtained from two generations of breeding of *B.t.* Cry1F maize line 1507, designated the T1S1 generation and the BC4 generation. The T1S1 generation seed consisted of the original transformed Hi-II line containing event TC1507 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 line containing event TC1507. Plants of both generations were grown in the greenhouse and leaf samples obtained for genomic DNA extraction and analysis.

Plasmid PHP8999 DNA, genomic DNA from Hi-II maize, and genomic DNA from *B.t.* Cry1F maize line 1507 T1S1 and BC4 generations were digested with the restriction enzymes *Pme* I, *Hin*d III, *Pst* I, *Bam*H I, *Eco*R I, and *Bam*H I combined with *Eco*R I. Genomic DNA lots within each *B.t.* Cry1F 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. The digestions with *Hin*d III, *Pst* I, and *Bam*H I were conducted for the purpose of characterizing the *cry*1F gene and its ubiquitin promoter in event 1507. The *Hin*d III restriction enzyme cuts at the 5' end of the ubiquitin promoter and the 3' end of the *cry*1F coding sequence. The purpose of the *Hin*d III digestion is to determine whether the full length *cry*1F gene is present with its promoter intact. The *Pst* I digestion is intended to provide further information about whether the ubiquitin promoter is intact as this enzyme cuts essentially at both ends of this promoter. *Bam*H I digestion is intended to provide information on whether the *cry*1F coding sequence is intact as this enzyme cuts at both the 5' and 3' end of this coding sequence.

The digestions with *Bam*H I, *Eco*R I, and the combination of *Bam*H I and *Eco*R I, were conducted for the purpose of characterizing the *pat* gene and its CaMV promoter in event 1507. The *Bam*H I enzyme cuts at the 5′ end of the *pat* gene and within approximately 150 bp of the 3′ end of this gene. The *Eco*R I enzyme cuts at the 5′ end of the CaMV promoter and in the CaMV terminator for the *pat* gene. Both digestions are intended to provide information on the presence of an intact copy of the *pat* gene and its CaMV promoter. An additional digestion with *Bam*H I and *Eco*R I was conducted to determine whether a 546 bp fragment corresponding to the CaMV promoter could be detected after hybridization with the CaMV DNA probe. *Pme* I digestion was conducted with the intent of isolating the entire linear insert used to transform *B.t.* Cry1F maize line 1507.

The expected results for each digestion and hybridization are shown in Table 3. 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 *B.t.* Cry1F maize line 1507 was inserted into the genome as a single intact copy.

Results and Conclusions

The results of the detailed molecular characterization of event TC1507 supports the conclusion that this event contains a full-length copy of the DNA insert used to transform this maize line (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 event TC1507.

The conclusion that a full-length copy of insert DNA PHI8999A is present in event TC1507 is based on the results of several hybridization experiments. The expected results for the *cry*1F gene can be summarized as follows. *Hin*d III digestion is expected to produce a 3890 bp fragment containing the ubiquitin promoter and *cry*1F gene. This expected fragment was observed after hybridization with probes specific for the ubiquitin promoter and *cry*1F (Fig. 5 and 6). A conclusion that the ubiquitin promoter is intact is supported by the *Pst* I digestion, which resulted in the expected 1986 bp fragment when genomic DNA was hybridized with the *ubi* probe (Fig. 6). Finally, the expected 1828 bp fragment was present when genomic DNA was digested with *Bam*H I and hybridized with the *cry*1F probe, which indicated an intact *cry*1F coding sequence is present (Fig. 5). Table 4 summarizes these results for the *cry*1F gene and its promoter.

The results for the *pat* gene can be summarized as follows. The *Eco*R I enzyme cuts at the 5′ end of the CaMV promote and in the CaMV terminator for the *pat* gene and is expected to result in a 1329 bp fragment if an intact copy of the *pat* gene and its CaMV promoter is present in TC1507. The 1329 bp fragment was observed after hybridization with the *pat* and CaMV DNA probes (Fig. 7 and 8). The presence of an intact *pat* gene was confirmed because the expected fragments were observed after *Bam*H I digestion followed by hybridization with the *pat* DNA probe (Fig. 7). The presence of an intact CaMV promoter was confirmed because the expected 546 bp fragment was observed with *Bam*H I and *Eco*R I digestion (Fig. 8). Finally, *Hin*d III digestion is expected to produce 2170 bp fragment containing the CaMV promoter and the *pat* gene, if both genes are present as full length copies. This expected fragment was observed after hybridization with the *pat* and CaMV DNA probes (Fig. 7 and 8). Table 4 summarizes these results for the *pat* gene and its promoter.

Digestion with *Pme* I was also conducted in an attempt to release the entire sequence for insert DNA PHI8999A. However, no bands of the expected 6235 bp size were observed, most likely as a result of loss of the *Pme* I sites either at the 3′ end, the 5′ end or both ends of the PHI8999A DNA insert (Table 4). It is commonly observed that plant cellular nuclease activity causes limited digestion at the exposed ends of DNA inserts used to produce transformation events.

The evidence for an additional copy of the *cry*1F sequence is based on results of the *Hind* III and *Pst* I digestions followed by hybridization with the *cry*1F and ubiquitin probes as shown in Table 4. *Hind* III digestion and hybridization with the *cry*1F probe resulted in two bands: one of the expected 3890 bp size and a second, representing the additional copy, that is larger and estimated at ~4000 bp in size (Fig. 5). (A third band of approximately 1000 bp is present, however, this likely is due to non-specific hybridization as a similar band is observed in the negative control.). Hybridization of the *Hind* III digest with the ubiquitin probe resulted in one band of the expected 3890 bp size and failed to reveal the ~4000 bp fragment (Fig. 6). This indicates that the promoter region is either absent in this additional copy or it is not intact. A small portion of the ubiquitin promoter cannot be detected by the ubiquitin DNA probe used in this study because the ubiquitin probe was prepared with a fragment of the ubiquitin promoter extending from 120 bp to 1707 bp (Table 2). Therefore, an approximately 300 bp region of the ubiquitin 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 ubiquitin promoter on the additional *cry*1F gene. Interpretation of hybridization results with the ubiquitin probe is made difficult by the fact that this promoter was isolated from maize and therefore is present in the nontransgenic control maize plants. This results in hybridizing bands that appear in the lanes containing DNA from the nontransgenic control line and the transgenic lines (Fig. 6 and Table 4). In addition, there is some evidence for non-specific hybridization with this probe (see for example the results for the control for *Eco*R I and *Eco*R I/ *Bam*H I digestions in Fig. 6). Nevertheless, the results of the *Hind* III digestion support the conclusion that the ubiquitin promoter on the additional copy of the *cry*1F coding sequence is either absent or is not intact.

V.B. Confirmation of the absence of antibiotic resistance marker genes

The *Pme* I DNA fragment used to transform event TC1507 was obtained from plasmid PHP8999. The portion of the plasmid that was not used for transformation contained the kanamycin resistance gene, *nptII*. To verify that, as expected, *B.t.* Cry1F maize line 1507 does not contain the *nptII* gene, genomic DNA was hybridized with an *nptII* probe. The Southern blot is shown in Figure 9. No bands hybridizing to the *nptII* DNA probe were detected, which confirmed that the resistance gene is not present in *B.t.* Cry1F maize line 1507.

Table 2. Summary of DNA probe sizes and locations relative to plasmid PHP8999

DNA Probe	Probe size (bp)	Location on plasmid PHP8999 (bp to bp)	Comments
ubi	1587	120-1707	Hybridizes to the ubiquitin promoter for the *cry*1F gene
*cry*1F	979	2548-3527	Hybridizes to coding region for the c*ry*1F gene
CAMV 35S	438	4791-5229	Hybridizes to the CaMV 35S promoter for the *pat* gene
pat	309	5537-5846	Hybridizes to coding region for the *pat* gene
nptII	536	7497-8033	Hybridizes to coding region for the *nptII* gene for resistance to kanamycin

Table 3. Summary of expected hybridizing fragments.

	Expected Hybridizing Fragment Size in Base Pairs for each DNA Probe				
Restriction Enzyme	***ubi***	***cry*1F**	**CAMV 35S**	***pat***	***nptII***
Pme I	≥6235	≥6235	≥6235	≥6235	≤3269
Hind III	3890	3890	2170	2170	≤3444
Pst I	1986	914 944	1916	1916	≤3488
*Bam*H I	>2080	1828	1361	315 490	≤5510
*Eco*R I	>1467	3202	1329	1329	≤4919
*Bam*H I/*Eco*R I	>1467	1828	546	315 468	≤4897

Table 4: Summary of observed fragments.

	Observed Hybridizing Fragment Size in Base Pairs for each DNA Probe				
Restriction Enzyme	***ubi***	***cry*1F**	**CAMV 35S**	***pat***	***nptII***
Pme I	~23000*	~23000*	~23000	~23000	No fragment
*Hin*d III	~3890+ ~6500*	~ 1000* ~3890+ ~4000	~2170+	~2170+	No fragment
Pst I	~1986+ ~23000*	~914+ ~944+ ~6500	~1916+	~1916+	No fragment
*Bam*H I	~6000* ~9400* ~15000*	~1828+ ~8000	~1361+	~315+ ~490+	No fragment
*Eco*R I	~1700+ ~3000 ~3500 ~4000 ~9400	~3202+ ~23000	~1329+	~1329+	No fragment
*Bam*H I/*Eco*R I	~1700* ~4000* ~6500	~1828+ ~4000 ~8000	~546+	~315+ ~468+	No fragment

* Similar fragment observed in negative control genomic DNA
+ DNA fragment predicted based on sequence of plasmid PHP8999

Figure 5. Southern analysis of the *cry*1F gene in the DNA insert of event TC1507.

1: Non-transgenic control (5 µg)
2: Pooled DNA from the T1 generation (5 µg)
3: Pooled DNA from the BC4 generation (5 µg)
4: Plasmid PHP8999 control (9.5 pg, equivalent to 1 copy)
M: Molecular weight markers from *Hin*dIII digest of lambda DNA (1 µg)

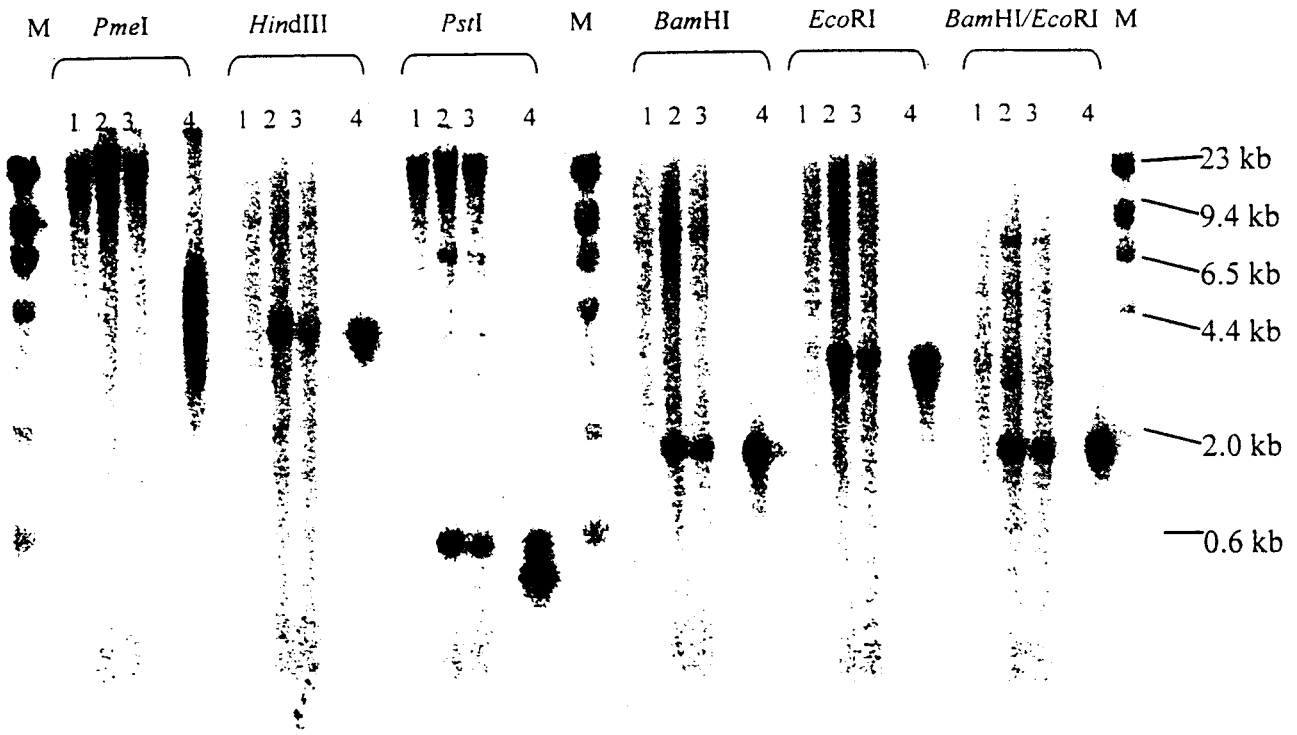


Figure 6. Southern analysis of the *cry*1F gene in the DNA insert of event TC1507.

1: Non-transgenic control (5 µg)
2: Pooled DNA from the T1 generation (5 µg)
3: Pooled DNA from the BC4 generation (5 µg)
4: Plasmid PHP8999 control (9.5 pg, equivalent to 1 copy)
M: Molecular weight markers from *Hin*dIII digest of lambda DNA (1 µg)

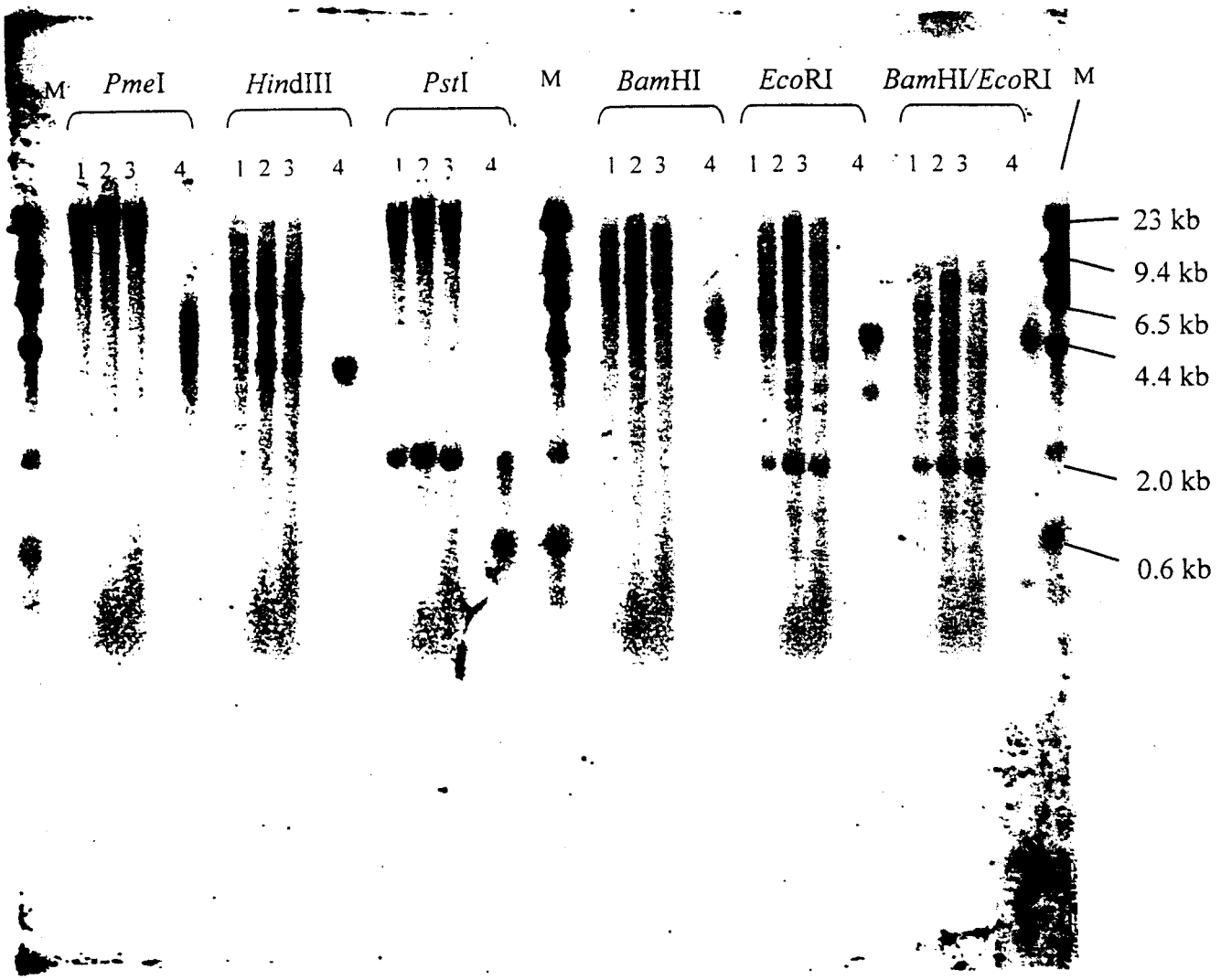


Figure 7. Southern analysis of the *pat* gene in the DNA insert of event TC1507.

1: Non-transgenic control (5 µg)
2: Pooled DNA from the T1 generation (5 µg)
3: Pooled DNA from the BC4 generation (5 µg)
4: Plasmid PHP8999 control (9.5 pg, equivalent to 1 copy)
M: Molecular weight markers from *Hin*dIII digest of lambda DNA (1 µg)

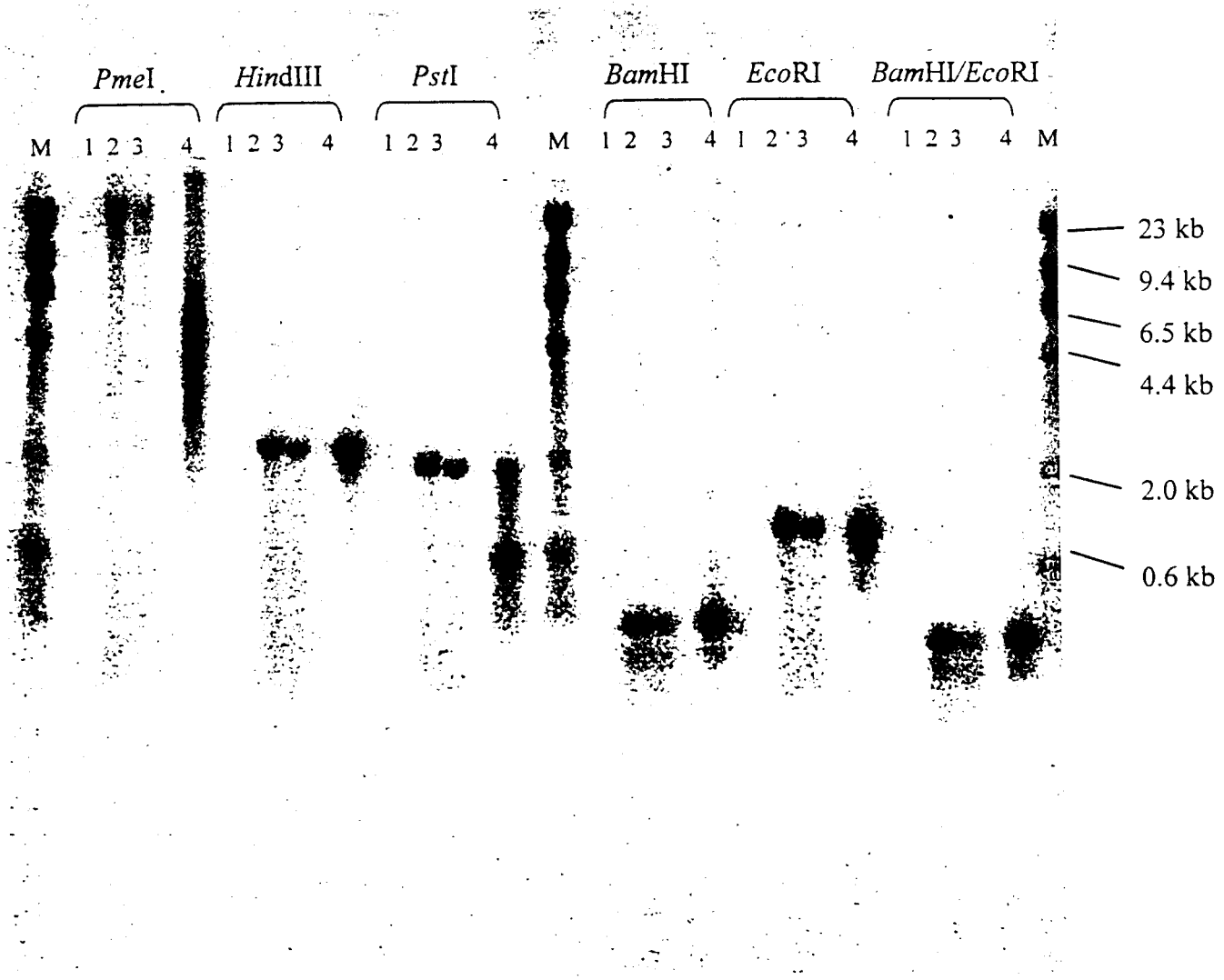


Figure 8. Southern analysis of the CaMV promoter for the *pat* gene in the DNA insert of event TC1507.

1: Non-transgenic control (5 µg)
2: Pooled DNA from the T1 generation (5 µg)
3: Pooled DNA from the BC4 generation (5 µg)
4: Plasmid PHP8999 control (9.5 pg, equivalent to 1 copy)
M: Molecular weight markers from *Hin*dIII digest of lambda DNA (1 µg)

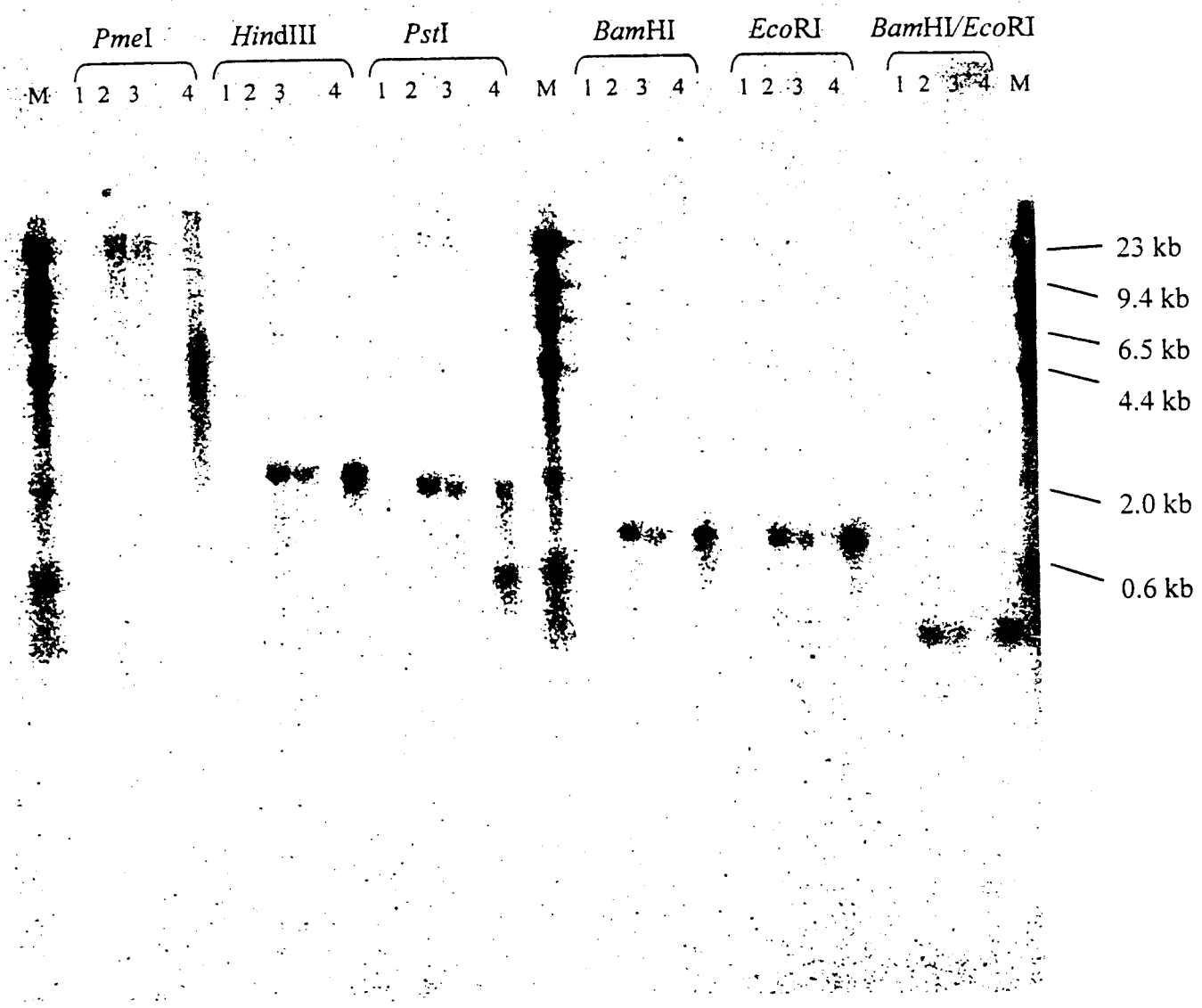
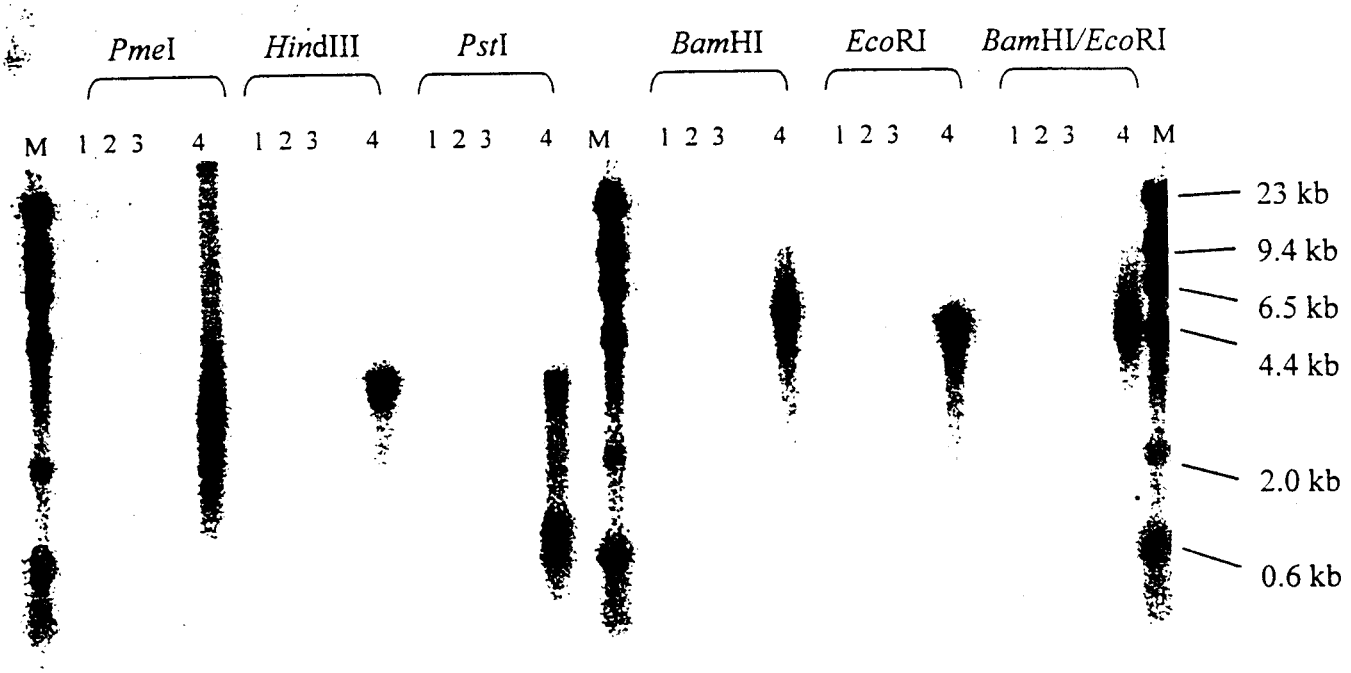


Figure 9. Southern analysis confirming the absence of the *nptII* gene in the DNA insert of event TC1507.

1: Non-transgenic control (5 µg)
2: Pooled DNA from the T1 generation (5 µg)
3: Pooled DNA from the BC4 generation (5 µg)
4: Plasmid PHP8999 control (9.5 pg, equivalent to 1 copy)
M: Molecular weight markers from *Hin*dIII digest of lambda DNA (1 µg)



V.C. Mendelian inheritance

Data on the Mendelian segregation of transgenes provides evidence of the stable inheritance of newly introduced genetic material. The Mendelian segregation of the *B.t.* Cry1F maize line 1507 was recorded and analyzed at two stages (see Fig. 10). The original transformed Hi-II line containing event TC1507 was crossed to an elite inbred to give an F1 hybrid. The F1 hybrid was backcrossed to the elite inbred one or two additional times to give BC1F1 or BC2F1 seed. Spraying at each generation eliminated glufosinate-susceptible plants and resulted in hemizygous seed.

Early segregation data was obtained by backcrossing the BC1F1 or BC2F1 hemizygous lines one additional time. The seed resulting from this backcross was planted, and the plants were sprayed with glufosinate. The expected segregation ratio was 1:1 (resistant:susceptible) for glufosinate tolerance. The BC2F1 – BC3F1 line of Table 5 details the results.

Later segregation data was obtained as follows: after one or two backcrosses, *B.t.* Cry1F maize line 1507 BC1F1 or BC2F1 seed was planted and self-pollinated. Resulting seed (BC1F2 or BC2F2) was expected to be 3 parts resistant and one part susceptible. It was planted and sprayed with glufosinate to remove the homozygous susceptible plants. The remaining plants (one part homozygous resistant and two parts hemizygous resistant) were crossed to a susceptible inbred to make F1 hybrid seed. This hybrid seed was planted and sprayed with glufosinate to check for the expected 2:1 resistant:susceptible ratio. Results are detailed in the F1 line of Table 5.

After the hybrids were sprayed with glufosinate and scored for resistance, 200 neonate European corn borers were used to infest each F1 plant that survived the glufosinate spraying. All of the plants determined to be tolerant to glufosinate were also found to be resistant to European corn borer infestation. These results support the conclusion that event TC1507 is a stable insertion event and is inherited as a Mendelian dominant gene. The Southern analysis results showing that the partial *cry*1F gene was present in the BC4 backcross generation supports the conclusion that it is genetically linked to the complete copies of *cry*1F and *pat* genes that are present in *B.t.* Cry1F maize line 1507.

Figure 10. Backcrossing generations used to determine Mendelian segregation ratios for *B.t.* Cry1F maize line 1507.

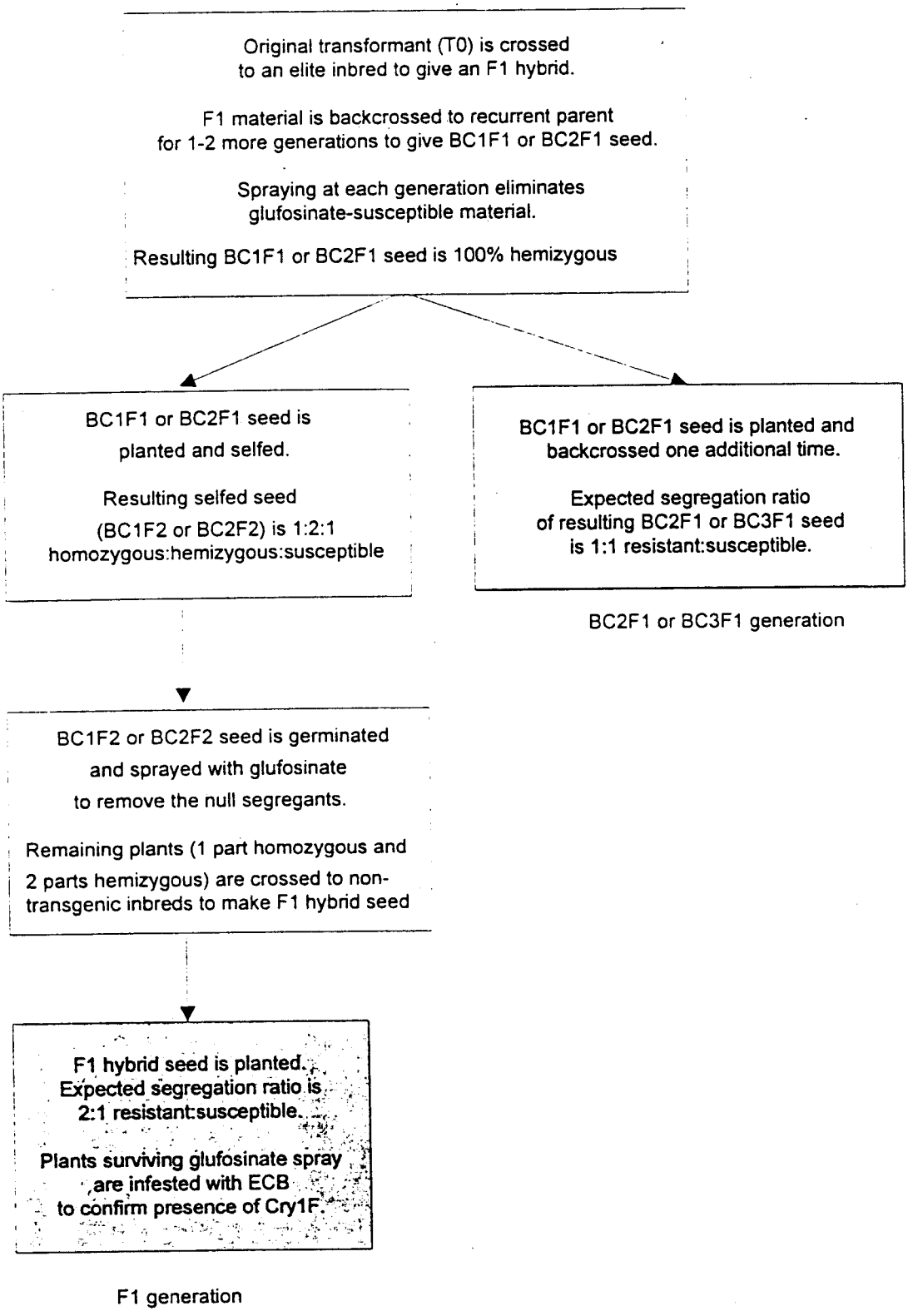


Table 5. Mendelian segregation of *B.t.* Cry1F maize line 1507.

Generation	Observed Ratio[1]	Expected Ratio	Chi Square	p-value	Significant Difference?[2]
BC2F1 - BC3F1	443 : 412	427.5 : 427.5	1.124	0.2891	No
F1	910 : 493	935.3 : 467.7	2.903	0.0884	No

[1] Data expressed as number of observed plants resistant to glufosinate: number of observed plants susceptible to glufosinate.
[2] Significant at alpha = 0.05

V.D. Expression of the Cry 1F and PAT proteins

A field study was conducted during the 1998/99 growing season in Chile to generate leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant tissue samples from a hybrid derived from *B.t.* Cry1F maize line 1507 and an equivalent hybrid control. Expression levels of Cry1F and PAT proteins in these tissues were measured using specific Enzyme Linked Immunosorbent Assay (ELISA) developed for each protein. The study was conducted at four field sites located in the major maize growing regions of Chile. The field sites were near the towns of Buin, Viluco, Graneros and Nancagua. These locations are comparable to regions in North America where the maize lines would be suitable commercial products.

All tissue samples were collected from a single replication of the hybrid test and control lines at each site. When the plants were at approximately the V9 (or nine leaf) stage of development the youngest whorl leaf was collected from five plants for the leaf sample. 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 silage stage (approximately four weeks after pollination), that were pooled at each site. The senescent whole plant samples, including ears, were harvested when all tissue had turned brown and dried.

The samples were lyophilized or oven dried and ground into a fine powder, then stored at –80°C until analysis by ELISA. For the ELISA analysis, samples were extracted in neutral pH buffer containing detergent (phosphate buffered saline plus Tween detergent). Total protein content of the samples was determined using the Bradford protein quantitation assay with bovine serum albumin as the protein in the standard curve. The standard curve and all samples were run in duplicate. The protein concentration of each sample was normalized so that the same amount of total protein was loaded in the ELISA. In this way, different signals in the ELISA can be attributed to different amounts of Cry1F in the sample and not simply a reflection of varying total protein loads.

A direct double antibody sandwich ELISA was utilized to quantify levels of Cry1F protein in genetically modified maize. The method uses a polyclonal rabbit antibody specific to Cry1F protein to capture the protein in the microtiter well. The captured protein is detected by the same polyclonal antibody conjugated to biotin. The binding of the biotinylated antibody to the captured protein was detected by a conjugate of streptavidin-alkaline phosphatase (SA/AP). The enzyme substrate, para-nitrophenyl phosphate (pNPP), was added for the color development. Standard curves were established using a series of Cry1F protein standards. The equation for the standard curve was calculated using the concentration of the standards and the corresponding mean absorbance readings of the standard. The expression levels of the samples were established based on correlating the absorbance reading of the sample to the standard curve. The Cry1F ELISA concentrations were expressed in pg/µg total protein.

The PAT ELISA assay is also a direct double antibody sandwich. The method uses a polyclonal rabbit antibody specific to the PAT protein and the SA/AP as described above. Again, quantification of the PAT protein was accomplished by extrapolation based on sample absorbance values from a standard protein concentration curve. The PAT ELISA concentrations were expressed in pg/µg of total protein.

On the ELISA reaction plates, duplicate wells were used for each sample and each sample total protein concentration was assayed in duplicate. Absorbance readings from the ELISA and total soluble protein determinations were recorded using the Bio-Rad Model 3550 plate reader. Cry1F and PAT protein expression means, standard deviations, and minimum/maximum values were calculated for each tissue type across locations. The results of the protein expression studies are summarized in Tables 6 and 7.

This study demonstrated that expression of the Cry1F protein was found at measurable levels in all test substance tissues sampled. Expression of the PAT protein was only found at measurable levels in the leaf tissue samples of the test samples. The Cry1F and PAT protein expression levels were below the limit of detection for all control substance samples.

Table 6. Summary of Cry1F protein levels in tissue collected from maize hybrid line 1507.

Tissue	Mean[1] Cry1F (pg/µg total protein)	Standard Deviation	Min/Max Range
Leaf	110.9	27.2	56.6 – 148.9
Pollen	135.5	13.5	113.4 – 168.2
Silk	50.3	16.5	26.8 – 79.8
Stalk	550.0	104.0	355.9 – 737.4
Whole plant	1063.8	361.7	803.2 – 1572.7
Grain	89.8	23.3	71.2 – 114.8
Senescent whole plant	714.3	95.5	622.2 – 845.3

[1] 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.

Table 7. Summary of PAT protein levels in tissue collected from maize hybrid line 1507.

Tissue	Mean[1] PAT (pg/µg total protein)	Standard Deviation	Min/Max Range
Leaf	<LOD[2]	NA[3]	<LOD – 40.8
Pollen	<LOD	NA	<LOD
Silk	<LOD	NA	<LOD
Stalk	<LOD	NA	<LOD
Whole plant	<LOD	NA	<LOD
Grain	<LOD	NA	<LOD
Senescent whole plant	<LOD	NA	<LOD

[1] 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.

[2] <LOD = below the limit of detection, LOD = <20 pg/µg total protein

[3] NA = not applicable.

V.E. Characteristics of the Cry 1F and PAT proteins as expressed in the plant

Western analysis techniques were utilized to determine if the Cry1F and PAT proteins expressed in the plant were of the same molecular weight and immunoreactivity as the native, microbially-expressed protein. Polyclonal antibodies were used that recognize multiple antigenic epitopes on the protein. 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 Cry1F or PAT protein.

B.t. Cry1F maize line 1507 leaf, pollen, grain and whole plant forage tissues from field grown plants were sampled for protein extraction. The samples were lyophilized or oven dried, ground into a fine powder, and extracted in neutral pH buffer containing detergent (phosphate buffered saline plus Tween detergent). Total protein content of the samples was determined using the Bradford protein quantitation assay with bovine serum albumin as the protein standard. Samples from negative control plants that do not express either the Cry1F or PAT protein were processed by the same methods.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) methods were utilized to denature the proteins during electrophoresis and allow maximum exposure of antigenic epitopes on the proteins. The protein extracts were also subjected to polyacrylamide gel electrophoresis under non-denaturing conditions that allow the proteins to remain in their normal conformation. The positive controls were either purified, microbially-expressed Cry1F protein or purified, microbially-expressed PAT protein. Gel electrophoresis was conducted with pre-cast Tris-Glycine polyacrylamide gels (4%-20%) using the Novex® Gel System according to manufacturer's instructions. Native or denaturing conditions were created by using a Tris-Glycine native sample and running buffer or a Tris-Glycine SDS sample and running buffer, respectively. Beta-mercaptoethanol was added to the sample buffer of the denaturing gel to create the necessary reducing conditions. Proteins were then transferred electrophoretically from the gel to a PVDF membrane for western blotting using tris-glycine transfer buffer. The Novex XCell II Blot Module and Mini-Cell apparatus were used for the transfer process. Western blots were performed using the Tropix® Western-Light chemiluminescent detection system according to manufacturer's instructions. The binding of the antibodies to the protein bands is detected by a chemiluminescent reaction.

The results of western analysis of Cry1F protein expression in plant tissues from *B.t.* Cry1F maize line 1507 are shown in Figure 11. Under denaturing conditions the Cry1F protein was detected as two bands of approximately 65 to 68 kD 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 the maize line 1507 tissues. Moreover, no immunoreactive proteins were detected in the negative control tissues, as expected, 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 figure, it has an apparent molecular weight that is slightly greater than the Cry1F protein. However, the western analysis conducted with Cry1F protein under non-denaturing conditions clearly shows that no immunoreactivity occurs with the Cry1F antibody in any of the negative control tissues (Figure 12). It can be concluded that the weakly reactive band observed under denaturing conditions for the grain negative control is due to possible binding of the Cry1F antibody to an epitope present on an endogenous maize protein.

The Cry1F protein detected in western analyses of *B.t.* Cry1F 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. 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). The N-terminal sequence of the full-length 68 kD Cry1F protein expressed *in planta* was blocked and therefore could not be sequenced. Therefore, 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 kD in its native form, comprised of two components of approximately 22 to 23 kD (Wehrmann *et al.*, 1996). The results of western analysis of PAT protein expression in plant tissues from *B.t.* Cry1F maize line 1507 are shown in Figure 13. PAT protein was detected by western analysis in leaf tissue of *B.t.* Cry1F maize line 1507 as a band of approximately 22 kD under denaturing conditions. No PAT protein was observed in pollen, whole plant, or grain of *B.t.* Cry1F maize line 1507. These results are consistent with the levels of PAT protein expressed in *B.t.* Cry1F maize line 1507 tissues (see Table 7) and the estimated detection limit of the western analysis procedure used in this study. 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 kD 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 kD PAT protein and a 43 kD form that did not denature during gel electrophoresis.

Figure 11. Immunoreactivity of the Cry1F protein expressed in tissues of *B.t.* Cry1F maize line 1507. Electrophoresis conducted under denaturing conditions.

Molecular weight standards from 4 kilodaltons (kD) to 250 kD are indicated on the figure.

Lanes are labeled as follows:

Leaf:	Maize line 1507 leaf tissue from two individual plants (23 µg and 55 µg of protein, respectively).
Leaf (-):	Leaf tissue from negative control maize line (41 µg of protein)
Pollen:	Maize line 1507 pollen tissue from two individual plants (41 µg and 61 µg of protein, respectively).
Pollen (-):	Pollen tissue from negative control maize line (26 µg of protein).
Whole plant:	Maize line 1507 whole plant tissue from two individual plants (12 µg and 6 µg of protein, respectively).
Whole plant (-):	Whole plant tissue from negative control maize line (14 µg of protein).
Grain:	Maize line 1507 grain tissue from two individual plants (82 µg and 98 µg of protein, respectively).
Grain (-):	Grain tissue from negative control maize line (79 µg of protein).
Cry1F protein:	Purified Cry1F protein

FIGURE 11

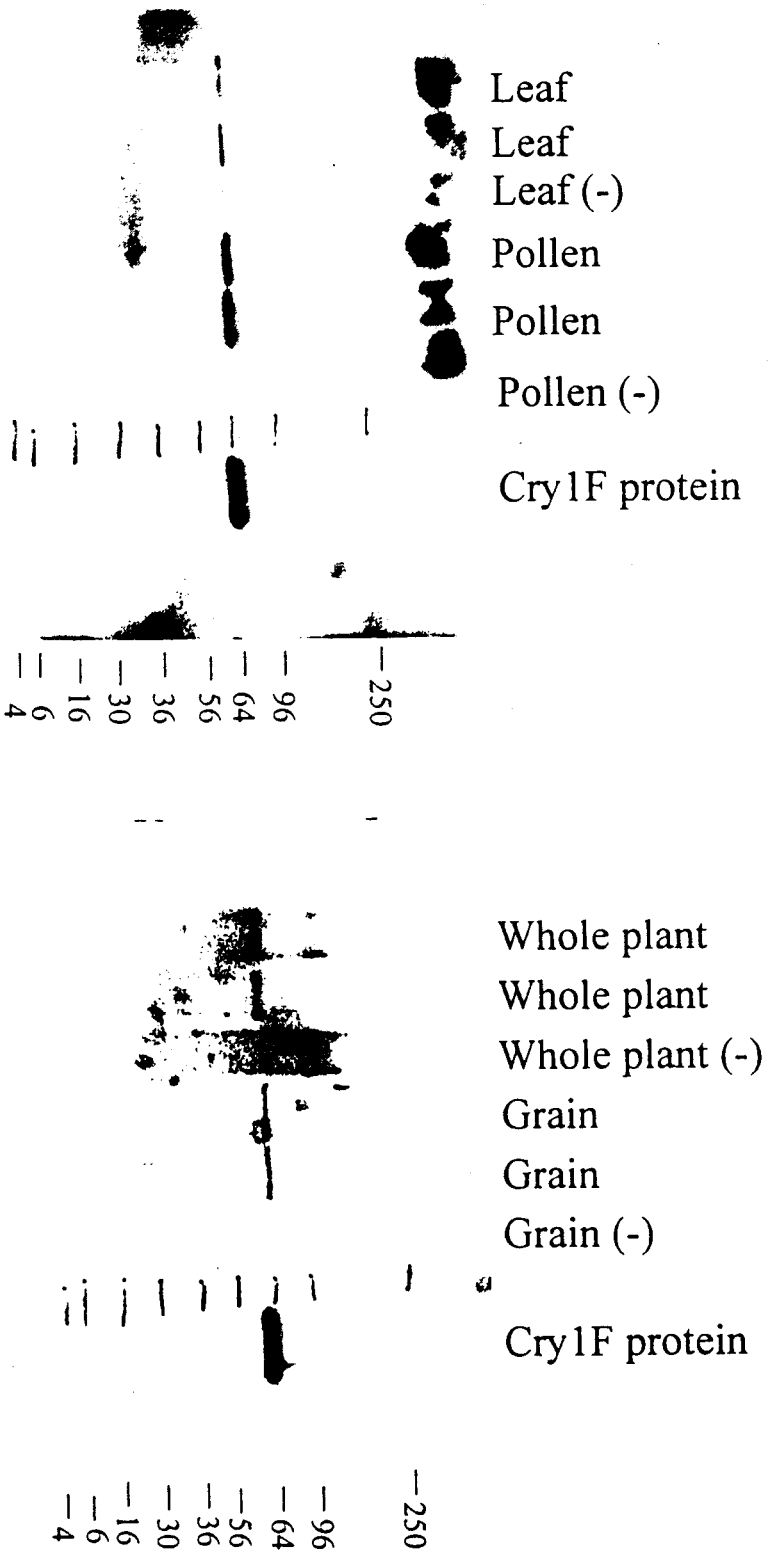

Leaf
Leaf
Leaf (-)
Pollen
Pollen
Pollen (-)
Cry1F protein
4
6
16
30
36
56
64
96
250
Whole plant
Whole plant
Whole plant (-)
Grain
Grain
Grain (-)
Cry1F protein
4
6
16
30
36
56
64
96
250

Figure 12. Immunoreactivity of the Cry1F protein expressed in tissues of *B.t.* Cry1F maize line 1507. Electrophoresis conducted under non-denaturing conditions.

Molecular weight standards from 4 kilodaltons (kD) to 250 kD are indicated on the figure.

Lanes are labeled as follows:

Leaf:	Maize line 1507 leaf tissue from two individual plants (23 µg and 55 µg of protein, respectively).
Leaf (-):	Leaf tissue from negative control maize line (41 µg of protein)
Pollen:	Maize line 1507 pollen tissue from two individual plants (41 µg and 61 µg of protein, respectively).
Pollen (-):	Pollen tissue from negative control maize line (26 µg of protein).
Whole plant:	Maize line 1507 whole plant tissue from two individual plants (12 µg and 6 µg of protein, respectively).
Whole plant (-):	Whole plant tissue from negative control maize line (14 µg of protein).
Grain:	Maize line 1507 grain tissue from two individual plants (82 µg and 98 µg of protein, respectively).
Grain (-):	Grain tissue from negative control maize line (79 µg of protein).
Cry1F protein:	Purified Cry1F protein

FIGURE 12

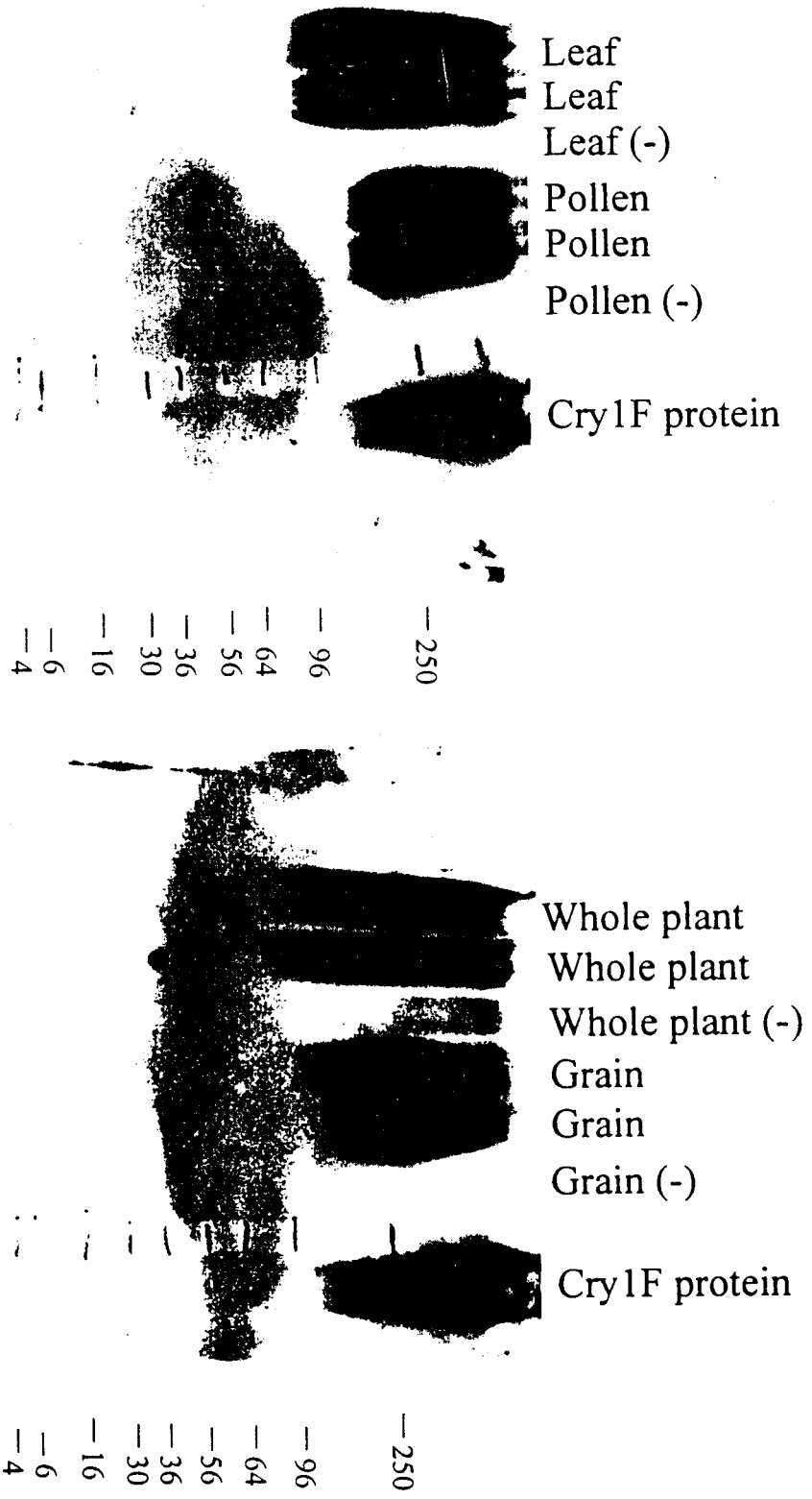

Leaf
Leaf
Leaf (-)
Pollen
Pollen
Pollen (-)
Cry1F protein
250
96
64
56
36
30
16
6
4
Whole plant
Whole plant
Whole plant (-)
Grain
Grain
Grain (-)
Cry1F protein
250
96
64
56
36
30
16
6
4

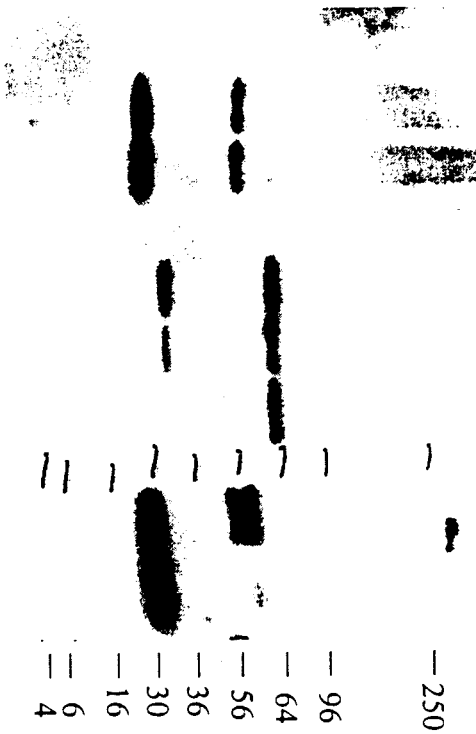
Figure 13. Immunoreactivity of the PAT protein expressed in tissues of *B.t.* Cry1F maize line 1507. Electrophoresis conducted under denaturing conditions.

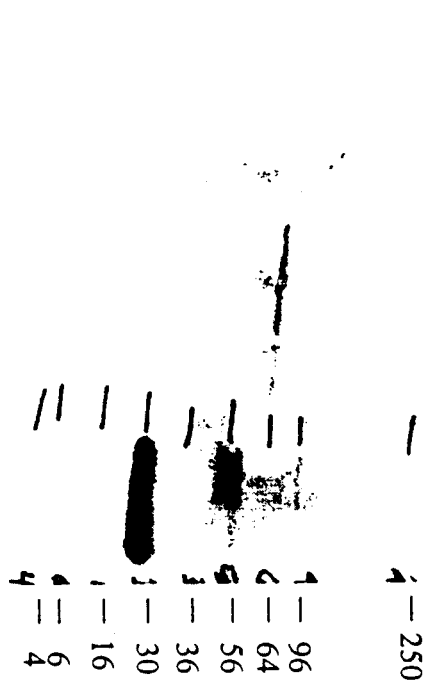
Molecular weight standards from 4 kilodaltons (kD) to 250 kD are indicated on the figure.

Lanes are labeled as follows:

Leaf:	Maize line 1507 leaf tissue from two individual plants (23 µg and 55 µg of protein, respectively).
Leaf (-):	Leaf tissue from negative control maize line (41 µg of protein)
Pollen:	Maize line 1507 pollen tissue from two individual plants (41 µg and 61 µg of protein, respectively).
Pollen (-):	Pollen tissue from negative control maize line (26 µg of protein).
Whole plant:	Maize line 1507 whole plant tissue from two individual plants (12 µg and 6 µg of protein, respectively).
Whole plant (-):	Whole plant tissue from negative control maize line (14 µg of protein).
Grain:	Maize line 1507 grain tissue from two individual plants (82 µg and 98 µg of protein, respectively).
Grain (-):	Grain tissue from negative control maize line (79 µg of protein).
PAT protein:	Purified PAT protein
Data not used:	Data generated for purposes other than this study.

FIGURE 13


Leaf
Leaf
Leaf (-)
Pollen
Pollen
Pollen (-)
PAT protein
Data not used
4
6
16
30
36
56
64
96
250


Whole plant
Whole plant
Whole plant (-)
Grain
Grain
Grain (-)
PAT protein
Data not used
4
6
16
30
36
56
64
96
250

V.F. Disease and pest resistance characteristics

B.t. Cry1F maize line 1507 has been field tested since 1997 in the major maize growing regions of the United States as well as in Puerto Rico and Hawaii (see Appendix 1). The breeders conducting each field test visually monitored disease (Northern leaf blight, Southern leaf blight, Southern rust, grey leaf spot, Stewart's wilt, smut) and pest resistance (corn earworm, fall armyworm, thrips, aphids, rose beetles, corn flea beetles, red spider mites, European corn borer) characteristics of *B.t.* Cry1F maize line 1507 and non-modified maize lines. There were no differences reported in severity of disease symptoms or insect damage (other than the targeted organisms susceptible to the Cry1F protein) between the plants from *B.t.* Cry1F maize line 1507 and non-modified maize lines.

V.G. Agronomic characteristics

Field trials of a hybrid derived from *B.t.*Cry1F maize line 1507 and an appropriate control hybrid were established across key maize growing regions of the U.S. in 1999. A summary of agronomic trait data taken from those trials is provided in Table 8. Expression of the Cry1F protein in maize lines inhibits feeding by European corn borer and certain other lepidopteran insect pests and protects against yield loss. The results support the conclusion that there are no agronomic differences between *B.t.* Cry1F maize line 1507 and non-modified hybrids.

B.t. Cry1F maize line 1507 seed was also tested for germination under both cold and warm growing conditions. The cold test is a stress test on seed simulating early spring planting conditions in the Midwestern US. Field soil is spread in a thin layer over a wet germination towel and chilled overnight. The following day, hybrid test seeds (e.g., *B.t.* Cry1F maize line 1507) are placed into the soil. The test seed is placed in a 10°C (50°F) chamber for seven days. After seven days, the test seed is moved to a 25°C (78°F) chamber for a three-day growout. The average percentage of germination of the test seed is compared to seed from four hybrids grown under the same conditions. The warm test is a germination test required by federal regulations governing seed quality and it is conducted under optimal growing conditions. Test seed is placed between sheets of germination toweling. The towels are then rolled inside a sheet of polyethylene coated paper. The rolls are placed in a 25°C (78°F) chamber at 90% relative humidity for five days. After five days, the average percentage of germination is calculated and compared to standard germination percentages for maize.

The results of the cold germination test demonstrated that *B.t.* Cry1F maize line 1507 had germination percentages that were very similar to the control hybrid seed (Table 9). All of the *B.t.* Cry1F maize line 1507 seeds germinated in the warm germination test (Table 9), which is comparable to the range typically observed for maize seed germination of greater than 92% germination. The results show that *B.t.* Cry1F maize line 1507 is comparable to other maize hybrids in seed germination characteristics.

Table 8. Agronomic performance of *B.t.* Cry1F maize line 1507.

Trait	Hybrid Maize Line 1507	Near-isogenic hybrid	Number of locations	Number of reps	LSD[1]
Yield (bushels per acre)	183.4	178.0	15	41	7.5
Moisture (%)	18.8	18.6	15	41	0.07
Accumulated maize growing degree units to reach 50% pollen shed	1351	1353	4	12	18.4
Accumulated maize growing degree days to reach 50% silking	1343	1337	4	12	18.9
Grain density[2]	58.3	58.2	9	27	0.7
Plant height (inches)	99.3	98.4	9	19	2.7
Ear height (inches)	45.5	44.3	9	19	2.3
Early stand count establishment (average number of plants emerging per plot)	74.5	71.7	4	12	26.5
Visual rating of emergence vigor from spike to one-leaf stage[3]	6.1	6.0	4	12	0.6
Visual rating of vigor at three- to five-leaf stage[3]	6.1	6.3	4	12	0.6
Stalk lodging[4]	0.3	0.6	11	33	0.8
Root lodging[4]	1.1	1.5	10	30	1.4
Dropped ears per plot	0.0	0.0	10	30	0.1
Top integrity[5]	7.9	7.6	9	27	1.1

[1] Least Significant Difference at the 0.05 level
[2] Weight (in pounds) of a bushel of grain at 15.5% moisture
[3] 1-9 visual scale, with 9 being best
[4] Average number of plants per plot that showed lodging of the specified type
[5] 1-9 visual scale that describes how well the stalks remain intact above the ear, with 9 being best

Table 9. Germination of seed from *B.t.*Cry1F maize line 1507.

Cold germination[1]	
Maize Line 1507	**Control Hybrids**
88%	91%
Warm germination[1]	
Maize Line 1507	**Expected Range**
100%	92% to 100%

[1] Germination is expressed as the number of germinated plants/total plants X 100%

VI. Environmental Consequences of Introduction of the Transformed Cultivar

VI.A. Estimated environmental concentrations

Estimated environmental concentrations (EEC) were calculated to conservatively represent worst case upper bounds of Cry1F protein exposure for comparison against ecotoxicity endpoints. Field protein expression studies (section V.D.) show average values of 1064 pg Cry1F per µg protein in whole plants at silage stage. At senescence, the average Cry1F protein concentration is 714 pg Cry1F per µg protein in whole plants.

In order to convert concentrations of Cry1F protein determined on a pg per µg total protein basis into EEC for whole plant tissue and environmental matrices (soil and water), estimates of plant biomass, dry matter (DM) content, protein content, and leaf area are needed. The upper bound estimates for biomass production are based on assumptions of maize grain yield of 235 bu $acre^{-1}$ and of stover as 65% of total above ground biomass (Martin *et al.*, 1976). The biomass estimates are highly conservative based on representative maize yields for the US and Ontario, Canada of 123 and 90 bu $acre^{-1}$, respectively (USDA, 1997; Statistics Canada, 1997). From this information, estimates for silage and stover biomass are 28 and 15 tons per acre for biomass containing 65 and 85% DM, respectively. The 65 and 85% DM values represent standard dry matter contents for silage and stover, respectively (Morrison, 1961). An upper bound estimate for total protein content of maize at silage stage is 0.9% (whole plant basis) and for standing stover at maturity is 0.6%. A leaf area index of 4 was assumed for conversion of *B.t.* toxin concentrations from a mass per mass basis to a mass per surface area basis.

The EECs for plant material are:

Standing green biomass (whole plants at silage stage):	15 µg Cry1F (g @ 65% DM)$^{-1}$ 1483 ng Cry1F cm^{-2} leaf area
Post-harvest stover (whole plants at senescence):	350 ng Cry1F (g @ 85% DM)$^{-1}$
Grain at harvest:	7 µg Cry1F (g @ 85% DM)$^{-1}$

These EEC are indeed conservative as shown by the comparison of the EEC for grain (7 µg Cry1F g^{-1}) with the measured Cry1F protein concentration in maize grain of *B.t.* Cry1F maize line 1507 (2.2 to 3.5 µg Cry1F g^{-1}; Young and Herman, 1999).

In order to determine a worst case EEC for plant-derived Cry1F protein concentrations in soil, the post-harvest stover was assumed to be incorporated to a 6-inch depth for a soil of average bulk density (2×10^6 lbs $acre^{-1}$ to a 6-inch depth).

Stover returned to soil:	0.125 lb Cry1F per acre 0.063 mg Cry1F (kg soil)$^{-1}$

The EEC for Cry1F occurrence in surface water was estimated using the GENEEC farm pond scenario. The Cry1F toxin returned to the soil through incorporation to a 6-inch depth was assumed available for runoff/erosion to an edge of field pond in the two days immediately post-incorporation. Additional input assumptions were soil degradation rate based on bioavailable DT_{50} (3.13 days), no degradation in other environmental compartments, and maize Cry1F protein K_{OC} and solubility of 100 L kg^{-1} and 1000 g L^{-1}, respectively, as a conservative estimate.

Surface water exposure: 0.55 µg Cry1F L^{-1} (runoff from incorporated stover)
1.25 µg Cry1F L^{-1} (from pollen drift, below)

The EEC for off-target pollen deposition is based on upper bound estimates of pollen concentration (32 µg Cry1F per g fresh weight pollen) and off-target pollen deposition (134 lbs per acre) (Wolt, 1999). The off-target load of Cry1F protein from pollen transport is 0.0021 lbs per acre. Loadings of pollen to a standard pond at the field edge are conservatively projected to result in maximum water concentrations of 1.25 µg Cry1F L^{-1} from pollen drift. The run-off contribution from pollen is negligible.

VI.B. *B.t.* Cry1F protein equivalency

The toxicity of *B.t.* Cry1F protein to non-target organisms was examined in several studies using a microbial source of Cry1F protein as the test material (MR872). It is necessary to use a microbial source of Cry1F protein for certain toxicology studies due to limited quantities of the maize-expressed protein. Therefore the equivalence of the Cry1F protein from these two sources must be demonstrated to support the use of the microbially-derived protein in certain toxicology tests (Evans, 1998). Cry1F protein extracted from maize or from a microbial source was found to react similarly in a variety of biochemical (N-terminal amino acid sequence, molecular weight, immunoreactivity, and protein glycosylation) and biological tests. This equivalency supports the use of microbially-derived Cry1F protein in certain toxicology tests because the two proteins have similar biochemical and biological properties.

Molecular Weight and Immunoreactivity
Toxin extracted from transgenic maize demonstrated immunoreactive species corresponding to proteins of ~68 and ~65 kDa. The ~68 kDa species corresponds to the expected molecular weight of the gene product from the transgene inserted into maize. The ~65 kDa species was predominant, and corresponds to the expected product of limited N-terminal proteolysis, potentially arising during the purification procedure from plant material. The ~65 kDa species extracted from maize migrated in SDS-PAGE with a mobility corresponding to that of the trypsinized, microbially derived Cry1F and of authentic Cry1F standard (also trypsinized). There were no other immunoreactive species apparent in Western blots of Cry1F derived from maize. The material yielded a quantification of 0.158% toxin by ELISA (Evans, 1998).

Toxin extracted from a microbial source and truncated by trypsin revealed a predominant species corresponding to a protein of ~65 kDa by SDS-PAGE with Coomassie Brilliant Blue staining. This is the anticipated product of trypsinolysis of the Cry1F gene product. Contaminating bands in low abundance were observed by Coomassie staining at mobilities corresponding to ~100, 45, 20 and 15 kDa. Western blotting demonstrated a predominat immunoreactive species at a mobility corresponding to a protein of ~65 kDa, plus other immunoreactive species at mobilities corresponding to ~25 and ~20 kDa. This material yielded a quantification of 11.4% toxin by SDS-PAGE. ELISA was not attempted due to the low molecular weight immunoreactive species (Evans, 1998).

N-terminal Sequence Analysis

Though the N-terminus of plant-derived Cry1F was blocked significantly, a sequence was able to be deduced by inspection of the chromatograms. A five amino acid sequence corresponding to the expected N-terminus of proteolytically cleaved Cry1F was obtained. The observed plant derived sequence was "^{28}STGRL", where the superscript refers to the amino acid residue in the subject protein. This corresponds to the expected N-terminus following cleavage by a trypsin-like activity ("^{20}VEILNEER$^{\Downarrow 28}$STGRLPLDI"). For the microbial derived Cry1F three N-terminal sequences were deduced; 1)"^{28}STGRLPL", 2) ^{33}PLDI" and 3) "^{43}FLLSEFV". The first N-terminal sequence matches the predicted sequence in plant derived material. The second represents a fragment between the first and third N-terminal sequences. And the third N-terminal sequence beginning at ^{43}F, represents cleavage at a 2nd trypsin recognition site ("^{39}SLTR$^{\Downarrow 43}$FLLSEFVP") (Evans, 1998).

Glycosylation

The immunoblot detection technique used in this study demonstrated that there was no apparent post-translational modification of Cry1F in maize-derived extracts involving carbohydrates. Appendix I, Figure 4B, lanes 8 and 10, shows that the bacterially-derived Cry1F materials are essentially bereft of reactivity. The maize-derived materials exhibit a number of discrete molecular weight species (Evans, 1998; Appendix I, Figure 4B, lanes 4 and6), however, none of these co-migrate with mobilities concordant with the immunoreactive species obtained in the western blot (Evans, 1998; Appendix I, Figure 3).

Biological Activity

Bioassays were conducted on sensitive and insensitive insects to compare the biological activity of maize-expressed Cry1F protein and microbially-derived Cry1F protein by (Evans, 1998). Sensitive insects used were European corn borer, tobacco budworm and fall armyworm. Insensitive insects used were black cutworm and corn earworm. The bioassays employed a diet surface overlay technique, sometimes called a "top-load" delivery of protein. Dipel™ at a 2X rate was used as a positive control. One dose was tested in 48 wells infested with one neonate larva per well. Mortality was assessed after six days for determination of LC_{50}. Three replications of each test substance/ species combination were tested over time although some exceptions were made. If negligible mortality occurred (defined as less than 15%) in either of the first two runs, a third run was not carried out (i.e. for control plant assays). Two types of controls were initially carried out for each bioassay. These were 1) untreated control consisting of only phosphate buffer plus 0.1% BSA and 2) tissue blanks of lyophilized maize plant tissue from control plants. The maize tissue blanks were formulated to contain the same amount of test material as was tested in the maize-derived Cry1F protein bioassays.

The results of the top-load bioassays indicated the two test materials performed indistinguishably on susceptible species in bioassays normalized by reported protein content (Table 10). Clear distinctions for non-susceptible species were not possible due to the very low activity of Cry1F on these non-target species. The rank order (LC_{50}) of susceptibility for species tested for both protein preparations was:

MOST SUSCEPTIBLE

⇓

European Corn Borer > Tobacco Budworm

Tobacco Budworm = Fall Armyworm

Fall Armyworm >> Corn Earworm

Corn Earworm > Black Cutworm

⇓

LEAST SUSCEPTIBLE

These results were confirmed in a dietary incorporation study with European corn borer that utilized a top-load bioassay for LC_{50} determinations (Table 10; Evans, 1998). The results of the top-load bioassays with the positive control, Dipel™, showed the validity of the bioassay system. Highly significant dose-response lines were generated against all five insect species. The rank order of sensitivity was shown to be the same for both proteins.

Table 10. Biological activity of maize-derived Cry1F protein and microbially-derived Cry1F protein.

	Top Load Bioassays			Diet Incorporation Bioassay
Source of Cry1F protein	LC_{50}, ng Cry 1F per cm^2			LC_{50}, ppm Cry 1F
	European Corn Borer	Tobacco Budworm	Fall Armyworm	European Corn Borer
Maize-derived Cry1F	0.58	1.74	2.21	0.11
Microbially-derived Cry1F	0.58	1.88	2.49	0.14

VI.C. Exposure to non-target organisms

Environmental fate of Cry1F protein incorporated into soil

The time-dependent loss in bioavailability of Cry1F protein following incorporation into a typical maize-growing soil was determined under laboratory conditions (Halliday, 1998). Test treatments consisted of Cry1F and DiPel™ (as a positive control), with and without soil, as well as untreated soil as a negative control. Soil amended with 18.1 ng microbially-derived Cry1F protein per g soil (oven dry equivalent) was incubated at 75% of field capacity and 24°C for up to 28 days (4.05 g moist soil per treatment). Samples removed at 0, 3, 5, 7, 10, 14 and 28 days following amendment were immediately frozen at – 70°C until prepared for bioassay. For each bioassay, treatments were suspended in 20 ml of 0.2% agar solution. The bioassay measured growth inhibition of *Heliothis virescens* first instar larvae following exposure to a series of doses in a diet incorporation assay. For each treatment/exposure length combination, an estimated EC_{50} (volume of the solution causing a 50% reduction in growth) was calculated. Non-linear regression of these data was used to determine the estimated time for 50% reduction in bioavailability (DT_{50}).

The results of this study indicated that soil-applied Cry1F protein exhibited a greater than 20-fold decline in biological activity over the 28-day test period. The estimated DT_{50} was 3.13 days. These results are consistent with those for Cry1A(b) protein as reported by Sims and Holden (1996). In their study, using essentially the same experimental design, a soil DT_{50} of 1.6 days was reported for the Cry1A(b) protein.

Chronic exposure of *Folsomia candida* (Collembola) to microbially-derived Cry1F protein

A 28-day study to determine the chronic effects of microbially-derived Cry1F protein on survival and reproduction of Collembola was conducted (Halliday, 1998). Three treatment levels of the Cry1F test substance (0.63, 3.1, and 12.5 mg kg^{-1}), as well as the reference substance (thiodicarb) were blended with Brewer's yeast to prepare the test diet. The treatment levels of Cry1F protein in this study were from 22- to 438-fold higher than would be encountered in the field, based on the exposure estimates calculated for Cry1F protein in soil. Bioassays were conducted on the Cry1F protein test diet at the start and conclusion of the experiment to verify bioactivity. The assay control was 100% yeast.

At the conclusion of the test, there was less than 10% mortality associated with exposure to either the Cry1F protein test substance or the assay control. Dose-dependent mortality was seen for the reference substance (thiodicarb) with 100% mortality at the highest rate of 10,000 ppm. Reproduction of Collembola was not significantly affected by exposure to the test substance when compared to the assay control. The mean number of progeny was not significantly different from any of the test substance treatments. No mortality and no reduction in the number of Collembola progeny was observed following exposure to the test materials for 28 days. The results of this study indicate that at treatment levels expected to be found in maize fields, Collembola were not affected by chronic exposure to Cry1F protein.

Beneficial insect studies

Beneficial insect studies have been conducted to evaluate hazard to non-target insects. The table below summarizes data generated in studies using microbially-derived Cry1F protein. Measured effect levels are substantially higher than anticipated levels of Cry1F protein expression and exposure in the environment; therefore the risk is negligible.

Guideline	Study Title	Results
OPPTS* 885.4380	Acute Dietary Toxicity LD_{50} and/or NOEC – Honeybees	LD_{50} and/or NOEC ≥ 640ng Cry1F/larvae
OPPTS 885.4340	Non-target Insect – Green Lacewing	LC_{50} > 480 µg Cry1F/g diet
OPPTS 885.4340	Non-target Insect – Parasitic Hymenoptera	LC_{50} > 320 µg Cry1F/g diet
OPPTS 885.4340	Non-target Insect – Ladybird Beetle	LC_{50} > 480 µg Cry1F/g diet

*EPA Office of Prevention, Pesticides, and Toxic Substances Test Guideline numbers.

Additional non-target tests

Cry1F protein effects on *Daphnia magna*, earthworm, and bobwhite quail were evaluated in non-target tests. Exposure to Cry1F protein was as Cry1F maize pollen, soil amended with microbially-derived Cry1F toxin, and Cry1F maize grain for *D. magna*, earthworm, and bobwhite quail, respectively. Measured effect levels (LD_{50}, EC_{50}, or LC_{50}) are substantially higher than anticipated levels of Cry1F protein expression and exposure in the environment; therefore, the risk is negligible.

Guideline	Study Title	Results
OECD* 202	Acute Dietary Toxicity – *Daphnia magna*	48-hour EC_{50} > 100 mg Cry1F pollen/L
OECD 207	Acute Toxicity – Earthworm	LC_{50} > 2.5 mg Cry1F/kg dry soil
OPP 71-2 OECD 205	Acute Toxicity – Northern Bobwhite Quail	LC_{50} > 100,000 mg Cry1F maize grain/kg diet

*Organisation for Economic Cooperation and Development Test Guideline numbers.

Monarch butterfly exposure and risk

Risk associated with non-target exposure to maize pollen containing *B.t.* Cry1F δ–endotoxin was assessed for monarch butterfly (*Danaus plexippus*) larvae feeding on milkweed (*Asclepiadaceae spp.*) in the near-field edge of maize fields (Wolt, 1999). A deterministic screening level assessment based on worst case input assumptions considered exposure to monarch at the field-edge. Pollen dissemination to the field edge and EEC were empirically described on the basis of published data. This exposure assessment was subsequently modified to allow for a probabilistic analysis of uncertainties and sensitivities in the assessment.

Effects characterization was based on preliminary effects measurements for monarch larvae as well as the intergenera distribution of acute susceptibility for lepidopteran species. Interposition of log-probability plots for the LC_{50} of Cry1F protein to lepidopteran species with the exposure-distance plot for EEC in the near field zone showed limited intergenera risk. The slope of the exposure-distance plot for EEC indicated rapid fall-off in exposure with distance. Consequently, there is limited potential for non-target effects beyond the immediate field extremity. The EEC does not exceed the monarch no effect level (10 $\mu g\ g^{-1}$) at any point inside or outside of maize fields. The 90th percentile intergenera LC_{50} is not exceeded at field offsets greater than 0.2 meter.

Less than 1% of monarch larvae in the immediate 60 meters of the field edge are expected to occur within 1 meter of the field edge; therefore, exposure potential within the near field zone will be extremely limited. Maize pollen characterization and deposition patterns pose methodological uncertainties in this assessment (Wolt, 1999). However, recent field research supports the validity of the estimated environmental concentrations developed to date (Dively, 1999; Pleasents *et al.*, 1999; Sears, 1999).

Effects on endangered species

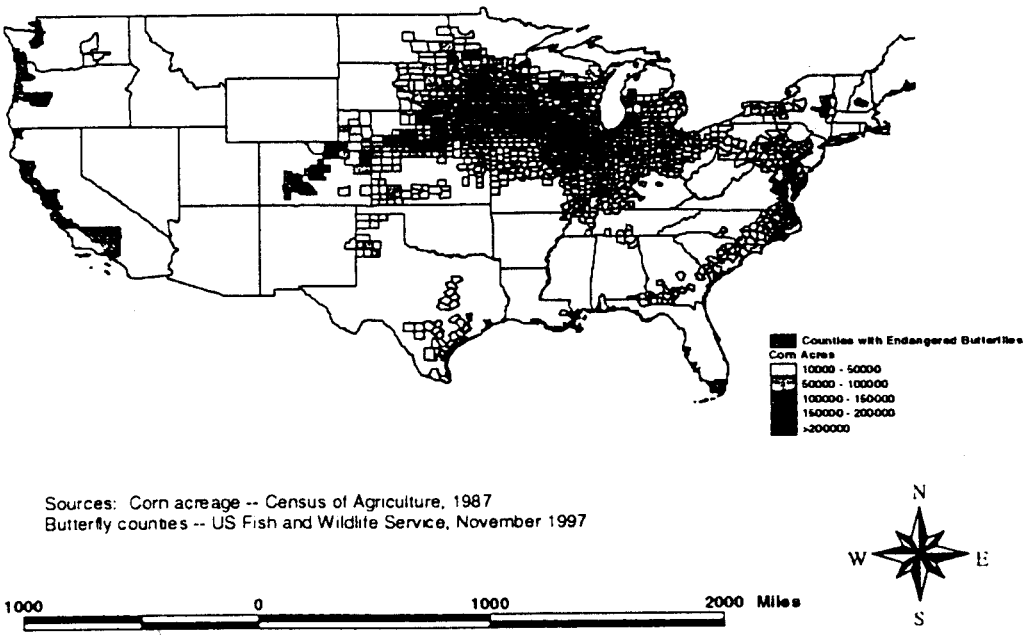
B. thuringiensis subspecies are differentiated by their insecticidal activity. Generally, only insect species within a given order (*Lepidoptera, Coleoptera, Diptera,* and *Hymenoptera*) are susceptible to a given insecticidal *B.t.* δ-endotoxin protein. Therefore, insect susceptibility results provide general information about the specificity of δ-endotoxin(s) expressed by particular *B. thuringiensis* strains. In the case of *B. thuringiensis* subspecies *aizawai* strains, the greatest activity is shown for the order *Lepidoptera.* Toxicological studies on non-target beneficial insect species using Cry1F maize pollen or microbially-expressed Cry1F protein show that insects of the order *Lepidoptera* are susceptible, given the margins of safety shown for representative species from other orders (*Hymenoptera, Diptera, and Coleoptera*).

The utility of maize-expressed Cry1F protein arises from its toxicity to lepidopteran pests of economic importance in maize production (e.g., European corn borer). Because *B.t.* δ-endotoxin is active against lepidopteran species, an assessment of the risk to non-target and endangered lepidopteran species is warranted. Assessment of risk to monarchs is a conservative predictor of risk to endangered lepidopteran species, since host-range specificity for endangered species further limits exposure potential. Thus, risk conclusions regarding monarchs may be bridged to a broader consideration of endangered lepidopteran species that may occur within the proximity of Cry1F maize production fields.

Endangered lepidopteran species currently listed by the U.S. Fish and Wildlife Service have very restricted host-range specificity. They do not occur in agricultural areas where maize is grown nor is maize considered a host plant for these species. Additionally, endangered species would have to consume Cry1F maize tissue (typically in the larval life stage) to be exposed to a potentially lethal dose. The risk to other endangered insect species is unlikely given the specificity of Cry1F protein for lepidopteran species and the low toxicity shown for non-target species.

The overlay map (Figure 14) describes the county level distribution of endangered lepidopteran species relative to maize production counties in the US. The map clearly indicates that any potential concern regarding range overlap with maize production is restricted to the Karner Blue butterfly. The Karner Blue butterfly has patchy distribution in the midwestern US in oak savannas and pine barrens. The Karner Blue butterfly feeds exclusively on wild lupine and as a result will not be exposed to maize-expressed *B.t.* protein.

Figure 14. **Map overlay of endangered butterfly distribution and maize acreage (by county)**



VI.D. Weediness of *B.t.* Cry1F maize line 1507

Weediness traits have been generally described by Baker (1974) as (1) the ability for weed seed to germinate in many different environments; (2) discontinuous germination and great longevity of seed; (3) rapid growth through vegetative phase to flowering; (4) continuous seed production for as long as growing conditions permit; (5) self-compatibility but partially autogamous and apometic; (6) ability to be cross-pollinated by unspecialized visitors or wind-pollinated; (7) high seed output in favorable environments and some seed production in a wide range of environments; (8) adaptation for short and long-distance dispersal; (9) vegetative production or regeneration from fragments and brittleness (hard to remove from the ground); and (10) ability to compete interspecifically by special means.

Maize does not exhibit any of the foregoing significant weedy tendencies and is non-invasive in natural environments (Canadian Food Inspection Agency, 1994). Maize hybrids have been domesticated for such a long period of time that the seeds cannot be disseminated without human intervention, nor can maize readily survive in the U.S. from one growing season to the next because of the poor dormancy. Volunteer maize plants are, in any case, easily identified and controlled through manual or chemical means.

The introduction of the trait for lepidopteran resistance should not confer a weediness trait to maize as protection from specific lepidopteran feeding would not increase the fitness capabilities of this maize line. Similarly, the trait for resistance to glufosinate herbicides has been used widely in plant breeding as a selective marker and for herbicide resistance in commercial products. The addition of this phenotypic trait to the *B.t.* Cry1F maize line described in this petition is not expected to increase fitness. As noted above, the survival of volunteer maize plants in the agricultural fields is easily controlled by manual or chemical means.

There are no wild, weedy relatives of *Zea mays* known to exist in the United States. Therefore, outcrossing of the *cry*1F gene or *pat* gene does not pose a plant pest risk due to the enhancement of weediness of wild relatives of maize

VI.E. Vertical transfer of the introduced genetic material

Non-cultivated *Zea mays* species are not found in the United States. The genus most closely related to *Zea* is *Tripsicum*, a genus of eleven species. Three *Tripsicum* species occur in the U.S. Crosses can be made between *Z. mays* and *T. dactyloides*, but these require human intervention and progeny are frequently sterile or genetically unstable. Therefore, cross-pollination between *Z. mays* and *T. dactyloides* in the natural environment is not expected to occur.

If outcrossing to cultivated maize should occur, the frequency with which this would occur is expected to be very low due to the short distances maize pollen will travel and the limited window of viability (Raynor *et al.*, 1972). Additionally, the outcrossing potential to cultivated maize is also diminished in seed production fields due to traditional containment practices to ensure seed genetic purity. Seed production fields are located in isolation to prevent introgression of genetic material from unwanted sources of maize pollen.

VI.F. Horizontal transfer of the introduced genetic material

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Even if such a transfer were to take place, transfer of the *cry*1F or *pat* gene from *B.t.* Cry1F maize line 1507 would not present a human health or plant pest risk. Genes encoding the PAT enzyme and similar acetyl transferases are found in nature. Similarly, the *cry*1F gene in *B.t.* Cry1F maize line 1507 was isolated from *Bacillus thuringiensis* var. *aizawai,* which is a ubiquitous soil bacterium that produces a variety of Cry proteins including Cry1F protein. Recipient *Bacillus thuringiensis* species would therefore not pose a greater plant pest risk than the wild type microbes from which the genes originated.

The *nptII* gene coding for resistance to the antibiotic kanamycin was demonstrated to not be present in *B.t.* Cry1F maize line 1507 (Fig. 9). The *Pme*I DNA fragment (designated insert PHI8999A) used to transform *B.t.* Cry1F maize line 1507 did not contain the *nptII* gene, although this gene was present on the original plasmid vector PHP8999.

VII. Adverse Consequences of New Cultivar Introduction

Bacillus thuringiensis (*B.t.*) is a diverse group of Gram positive, spore-forming bacteria best known as a valuable source of commercially important biopesticides. The most well studied class of *B.t.* insecticidal proteins is the δ-endotoxins. Advances in agricultural biotechnology have enabled the expression of *B.t.* toxins in transgenic plants, thereby imparting intrinsic insect resistance traits to a number of important crops (Prieto-Samsonov *et al.*, 1997). *B.t.* Cry1F maize line 1507 expresses the Cry1F protein at levels that provide effective control of European corn borer and other lepidopteran pests.

The evidence provided in this petition supports the conclusion that *B.t.* Cry1F maize line 1507 presents low risk to human health and the environment and does not present a plant pest risk. Based on exposure estimates and the results of toxicological studies, there is low risk to non-target organisms and beneficial insects from expression of the Cry1F protein in maize line 1507. *B.t.* Cry1F maize line 1507 exhibits typical agronomics and normal Mendelian inheritance of the introduced genetic material. There has been no evidence of increased susceptibility to insect pests or disease in *B.t.* Cry1F maize line 1507 when compared to conventional maize hybrids.

The commercial introduction of transgenic maize expressing the *B.t.* toxin has, for the first time, provided growers with a simple, cheap, highly effective, and environmentally benign means of controlling the European corn borer and other pests. This in turn has lead to a rapid adaptation of this technology by the agricultural community. The high rate of adaptation has resulted in fears that ECB will develop resistance to *B. thuringiensis* proteins when expressed in plants, despite the fact that ECB has not developed field resistance to any class of insecticides. Stable laboratory resistance to *B.t.* proteins at levels comparable to plant expression also has not been reported, despite many efforts to achieve this.

Hybrids derived from *B.t.* Cry1F maize line 1507 will be the first maize hybrids to use an insect control protein derived from *Bacillus thuringiensis* var. *aizawai.* As such, it demonstrates a different spectrum of activity against target lepidopteran pests. Field research trials with *B.t.* Cry1F maize line 1507 have indicated that Cry1F can control maize pests, such as fall armyworm, southwestern corn borer, and black cutworm, that have not previously been well controlled by other *B.t.* proteins expressed in plants. However, at least one insect, the diamondback moth (*Plutella xylostella* (Linnaeus)), has shown cross resistance between the Cry1A toxins and Cry1F (Tabashnik *et al.*, 1997). The cross resistance situation

for ECB cannot be predicted, but it can be conservatively assumed that Cry1F will share resistance with the Cry1A toxins in ECB. Further evaluation of laboratory reared insect colonies and studies on the binding affinity of the Cry1F protein will be necessary to address this issue. In order to reduce the probability that resistance will develop, Mycogen and Pioneer will continue to fund research to determine the sensitivity of wild ECB populations to Cry1F. These collections will be used to monitor for changes in the susceptibility of wild ECB populations. A refuge plan will be implemented during large-scale testing as well as future commercialization.

The *pat* gene and PAT protein in maize have been the subject of a previous determination of non-regulated status by the USDA (USDA, 1995). The subject maize events T14 and T25, developed by AgrEvo USA, contain the same synthetic version of the *pat* gene as contained in *B.t.* Cry1F maize line 1507. USDA determined that events T14 and T25: (1) exhibit no plant pathogenic properties; (2) are no more likely to become weeds than other maize developed by traditional breeding techniques; (3) are unlikely to increase the weediness potential for any other cultivated or wild species with which they can interbreed; (4) will not harm other organisms, such as bees, that are beneficial to agriculture; and (5) should not cause damage to processed agricultural commodities. Similarly, *B.t.*Cry1F maize line 1507 expressing the identical PAT protein is not expected to present any significant plant pest or environmental risk. The PAT protein has been shown to present no significant human health environmental risk based on acute oral toxicity studies and in vitro digestibility studies (EPA, 1997; EPA, 1995). In addition, glufosinate herbicide is registered for use on maize in the US.

VIII. References

Adang, M. J., Firoozabady, E., Klein, J., DeBoer, D., Sekar, V., Kemp, J.D., Murray, E., Rocheleau, T.A., Rashka, K., Staffeld, C., Stock, C., Sutton, D. and Merlo, D. J. 1987. Expression of a *Bacillus thuringiensis* insecticidal crystal protein gene in tobacco plants. Molecular Strategies for Crop Protection, C. Arntzen and C. Ryan, (Ed. Alan R. Liss) Inc. New York p. 345-353.

Baker, H.G. 1974. The Evolution of Weeds. Annual Review of Ecology and Systematics 5:1-24.

Barton, K.A., Whiteley, H.R., Yang, Ning-Sun, 1987. *Bacillus thuringiensis* δ-Endotoxin Expressed in Transgenic *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects, Plant Physiology 85:1103-1109.

Bosch, D., Schipper, B., van der Kleij, H., de Maagd, R., and Stiekema, J., 1994. Recombinant *Bacillus thuringiensis* Crystal Proteins with New Properties: Possibilities for Resistance Management. Bio/Technology 12: 915-918.

Canadian Food Inspection Agency. 1994. Regulatory Directive Dir 94-11: The Biology of *Zea mays* L. (Corn/Maize). CFIA, Plant Products Division, Plant Biotechnology Office, Ottawa.

Christensen, A.H., R.A. Sharrock, and P.H.Quail. 1992. Maize polyubiquitin genes: Structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant. Mol. Biol. 18:675-689.

De Maagd, R. A., Kwa, M. S. G., Van der Klei, H., Yamamoto, T., Schipper, B., Vlak, J. M., Stiekema, W. J., and Bosch, D. 1996. Domain III Substitution in *Bacillus thuringiensis* Delta-Endotoxin Cry1A(b) Results in superior Toxicity for *Spodoptera exigua* and Altered Membrane Protein Recognition. Appl. Environ. Microbiol. 62:1537-1543.

Dively, G.P. 1999. Deposition of corn pollen on milkweeds and exposure risk to monarch butterfly larvae in Maryland. Presentation at the Monarch Butterfly Research Symposium, November 2, 1999, Chicago, IL. Agricultural Biotechnology Stewardship Working Group.

Doebley, J.F. 1984. Maize introgression into teosinte – a reappraisal. Ann. Missouri Bot. Gard. 71:1100-1113.

Eckes, P., Vijtewaal, B., Donn, G. 1989. Synthetic gene confers resistance to the broad spectrum herbicide L-phosphinothricin in plants. J. Cell. Biochem. 13D:334.

EPA. 1995. Plant Pesticide Insert Ingredient Phosphinothricin Acetyltransferase (PAT) and the Genetic Material Necessary for its Production (Plasmid Vector pCIBP3064) in Corn; Tolerance Exemption. *Federal Register* 60:42450-42453.

EPA. 1997. Phosphinothricin Acetyltransferase and the Genetic Material Necessary for its Production in All Plants; Exemption from the Requirement of a Tolerance On All Raw Agricultural Commodities. *Federal Register* 62:17717-17720.

EPA. 1998. R.E.D. FACTS – *Bacillus thuringiensis*; EPA-738-F-98-001.

Evans, S.L. 1998. Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterization of Test Substances for Biochemical and Toxicological Studies. Report number MYCO98-001, an unpublished technical report by Mycogen Seeds c/o Dow AgroSciences.

Fischoff, D., Bowdish, K., Perlak, F., Marrone, P., McCormick, S., Niedermeyer, J., Dean, D., Kusano-Kretzmer, K., Mayer, E., Rochester, K., Rogers, S., and Fraley, R. 1987. Insect Tolerant Transgenic Tomato Plants. Bio/Technology 5:807-813

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J., Brousseau, R., and Cygeler, M. 1995. *Bacillus thuringiensis* Cry1A(a) Insecticidal Toxin: Crystal Structure and Channel Formation. J. Mol. Biol. 254:447-464.

Halliday, W.R. 1998. Environmental Fate of Cry1F Protein Incorporated into Soil. Report Number 7569-98-0080-AC-001, an unpublished technical report by Mycogen Seeds c/o Dow AgroSciences.

Kiesselbach, T.A. 1949. The Structure and Reproduction of Corn. Nebraska Agricultural Experimental Station Research Bulletin.

Klein, T.M., E.D. Wolf, R. Wu, and J.C. Sanford. 1987. High velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70-73.

Lee, M. K., Young, B. A., and Dean, D. H. 1995. Domain III Exchanges of *Bacillus thuringiensis* Cry1A Toxins Affect Binding to Different Gypsy Moth Midgut Receptors. Biochem. and Biophys. Res. Communications 216 (1):306-312.

Manglesdorf, P.C. 1974. Corn: its origin, evolution and improvement. p. 1-262. Harvard University Press; Cambridge, Massachusetts.

Martin, J. H., W. H. Leonard, and D. L. Stamp. 1976. Principles of Field Crop Production. 3rd ed. Macmillan, New York.

Morrison, F. B. 1961. Feeds and Feeding, Abridged. 9th ed. Morrison Publ., Claremont, Ontario.

Murray, E., Lotzer, J. and Eberle, M. 1989. Codon Usage in Plant Genes. Nucleic Acids Research 17(2):477-498.

Murray E.E., Rocheleau T., Eberle M., Stock C., Sekar V., Adang M. 1991 Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. Plant Mol. Biol. 16(6):1035-1050.

Nap, J.P., J. Bijvoet and W.J. Strikema. 1992. Biosafety of kanamycin-resistant transgenic plants: an overview. Transgenic Crops 1:239-249.

Odell, J.T., F. Nary, and N.-H. Chua. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810-812.

Pleasents, J.M., R. L. Hellmich, and L. C. Lewis. 1999. Pollen deposition on milkweed leaves under natural conditions. Presentation at the Monarch Butterfly Research Symposium, November 2, 1999, Chicago, IL. Agricultural Biotechnology Stewardship Working Group.

Prieto-Samsonov, DL., Vazquez-Padron, R.I., Ayra-Pardo, C., Gonzalez-Cabrera, J., and De la Riva, G. A. 1997. *Bacillus thuringiensis*: from Biodiversity to Biotechnology. J. Ind. Microbiol. Biotech. 19: 202-219.

Raynor, G.S., E.C. Ogden, and J.V. Hayes. 1972. Dispersion and deposition of corn pollen from experimental sources. Agronomy Journal 64:420-427.

Redenbaugh, K., W. Hiatt, B. Martineau, J. Lindemann and D. Emlay. 1994. Aminoglycoside 3'-phosphotransferase II (APH(3')II): review of its safety and use in the production of genetically engineered plants. Food Biotechnology 8:137-165.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., and Dean, D. H. 1998 *Bacillus thuringiensis* and Its Pesticidal Crystal Proteins. Microbiol. Mol. Biol. Reviews. Sept., 1998. p. 775-806.

Sears, M. 1999. Sensitivity and response of monarch larvae to *B.t.* corn pollen via bioassay. Presentation at the Monarch Butterfly Research Symposium, November 2, 1999, Chicago, IL. Agricultural Biotechnology Stewardship Working Group.

Sims, S.R. and Holden, L.R, 1996. Insect Bioassay for Determining Soil Degradation of *Bacillus thuringiensis* subsp. *kurstaki* Cry1Ab protein in Corn Tissue. Physio. Chem. Ecol. Vol. 25(3):659-664.

Smith, J.S.C., M.M. Goodman, C.W. Stuber. 1985. Relationships between maize and teosinte of Mexico and Guatemala: numerical analysis of allozyme data. Economic Botany 39:12-24.

Statistics Canada. 1997. Canadian Grain Corn Yields, 1908-1997. Statistics Canada and Ontario Ministry of Agriculture, Food and Rural Affairs.

Tabashnik, B., Malvar, TR., Liu, Y., Finson, N., Borthakur, K., Shin, M., Park, S., Masson, L., De Maagd, R., and Bosch, D. 1996. Cross-Resistance of the Diamondback Moth Indicates Altered Interactions with Domain II of *Bacillus thuringiensis* Toxins. Appl. Environ. Microbiol. 62 :2839-2844.

Tabashnik, B.E., Y.B. Liu, N. Finson, L. Masson, D.G. Heckel. 1997. One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins. Proc. Natl. Acad. Sci. 94:1640-1644.

USDA.1995. Availability of Determination of Nonregulated Status for Genetically Engineered Corn. Animal and Plant Health Inspection Service. 60(134):36095-36096.

USDA. 1997. Census of Agriculture, National Agricultural Statistics Service, United States Department of Agriculture.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M., and Leemans, J. 1987. Transgenic plants protected from insect attack. Nature 328:33-37

Van Wert, S. 1994. Petition for Determination of Nonregulated Status: Glufosinate Resistant Corn Transformation Events T14 and T25. U.S. Department of Agriculture, Washington, DC.

Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene, 33, 103-119.

Wehrmann, A., A. Van Vliet, C. Opsomer, J. Botterman, A. Schulz. 1996. The similarities of *bar* and *pat* genes products make them equally applicable for plant engineers. Nature Biotechnology 14:1274-1278.

White, P.J. and Pollak, L.M. 1995. Corn as a food source in the United States: Part II. Processes, Products, Composition, and Nutritive Values. Cereal Foods World 40:756-762.

Wolt, J. D. 1999. Non-target Exposure and Risk Assessment for Environmental Dispersal of Cry1F Maize Pollen. Report number GH-C 4988, an unpublished study of Dow AgroSciences.

Young, D. L. and Herman, R.A. 1999. Characterization of Expressed Cry1F Protein in maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures. Report number 990027, an unpublished study of Dow AgroSciences.

Dow AgroSciences LLC
5501 Oberlin Drive
San Diego, CA 92121

00-136-01f

Dow AgroSciences

Attachment 1: USDA Field Trial Approvals for *B.t.* Cry1F Maize Line 1507

USDA Notification #	Tracking #	Planting Dates	Acreage	Field Trial Location	Company
00-088-37n Permit still open report not yet submitted	MS132	5/13/00	21.8	Renville County, MN	Mycogen
		5/14/00	19.4		
		5/23-27/00	50.0	Dakota County, MN	
		5/24/00	23.0		
		5/11-25/00	19.88	Grundy County, IA	
		5/11-6/2/00	21.54		
		5/11-6/2/00	15.86		
		date pending	1.0		
		5/12/00	6.0	Story County, IA	
		5/25&31/00	18.3	Fillmore County, NE	
		5/22&29/00	13.4		
00-068-02n Permit still open report not yet submitted	MS123	5/4/00	0.02	Wilikin County, MN	Mycogen
		5/3/00	0.32		
		5/6/00	0.018	Renville County, MN	
		5/6/00	0.819		
		4/25/00	0.18	Winona County, MN	
		5/3/00	0.344	Jackson County, MN	
		5/2/00	0.5	Brown County, MN	
		5/3/00	0.046	Sac County, IA	
		5/3/00	0.046		
		5/2/00	1.38		
		4/27/00	0.05	Story County, IA	
		5/25/00	0.023		
		5/23/00	0.005		
		4/28/00	0.9		
		5/5/00	0.1		
		5/12/00	1.7		
		5/22/00	0.36		
		5/4/00	0.5	Plymouth County, IA	
		5/4/00	0.8	Marshall County, IA	
		4/29/00	0.6	Page County, IA	
		5/6/00	0.79	Scott County, IA	
		5/22/00	0.02	Boone County, IA	
		5/3/2000	0.275	Clay County, SD	
		4/28/00	0.1	Cass County, ND	
		5/4/00	0.1	Pierce County, WI	
		4/25/00	0.5		
		5/3/00	0.1	Columbia County, WI	
		5/15/00	0.011		
		5/15/00	0.011		
		5/5/00	1.0		
		5/5-11/00	0.8		
		4/29/00	0.6	Jefferson County, WI	
		5/23/00	0.18	Dane County, WI	
		4/26/00	.021	York County, NE	
		5/3/00	1.01		
		5/5/00	0.11	Dixon County, NE	
		5/1/00	1.1	Butler County, NE	
		5/9/00	0.2	Savoy, IL	
		5/17/00	0.6	Dekalb County, IL	
		6/7/00	0.6	Champaign County, IL	
		6/9/00	0.6		
		5/9/00	0.5		
		6/7/00	0.1		
		6/1/00	0.017	Massac County, IL	
		5/11/00	0.055	Macon County, IL	

1/16/01

Dow AgroSciences LLC
5501 Oberlin Drive
San Diego, CA 92121

00-136-04

Dow AgroSciences

USDA Notification #	Tracking #	Planting Dates	Acreage	Field Trial Location	Company
00-068-02n (continued) Permit still open report not yet submitted	MS123	5/2/00	0.95	Logan County, IL	Mycogen
		5/3/00	1.14	LaSalle County, IL	
		5/4/00	1.2	Knox County, IL	
		4/20/00	0.64	Tipton County, IN	
		4/29/00	0.0275	Benton County, IN	
		4/29/00	0.0275		
		9/01/00	0.0046		
		4/29/00	0.82		
		5/25/00	0.006	Tippecanoe County, IN	
		5/25/00	0.01		
		5/3/00	1.1	Decatur County, IN	
		5/5/00	0.22	White County, IN	
		5/4/00	0.037	Moore County, TX	
		4/24/00	0.0173	Swisher County, TX	
		dates pending	30.0	Santa Isabel, PR	
		11/22/00	0.07	Oahu County, HI	
		5/15/00	5.0	Maui County, HI	
		5/31/00		(total for all plantings	
		8/31/00		is 5 acres)	
		9/12/00			
		9/15/00			
		9/25/00			
		4/28/00	0.11	Haskell County, KS	
		4/28/00	0.048	Ford County, KS	
		5/8/00	0.1	Finney County, KS	
		5/25/00	0.022	Riley County, KS	
		5/02/00	0.01	Fayette County, KY	
		5/22/00	0.01		
		7/8/00	0.01		
		5/31/00	0.01	Caldwell County, KY	
		5/1/00	0.03	Callaway County, MO	
		6/7/00	0.03	Holt County, MO	
		5/10/00	0.06		
		date pending	0.1	Boone County, MO	
		date pending	0.1	Mississippi County, MO	
		4/24/00	0.137	Pike County, MO	
		5/4/00	0.2	Saline County, MO	
		5/30/00	0.027	Ingham County, MI	
		5/5/00	0.78	Lenawee County, MI	
		4/26/00	0.02	Washington County, MS	
		5/16/00	0.02		
		5/16/00	0.02		
		5/15/00	0.05	Tiff County, GA	
		5/15/00	0.2	Clark County, OH	
		5/15/00	0.02		
		5/11/00	0.17	Weld County, CO	
		5/11/00	0.17		
00-010-07n	MS121	5/11/00	0.03	Saunders County only, not planted in Lancaster, Dixon, or Clay Counties, NE	Mycogen

1/16/01

Dow AgroSciences LLC
5501 Oberlin Drive
San Diego, CA 92121

00-136-01

Dow AgroSciences

USDA Notification #	Tracking #	Planting Dates	Acreage	Field Trial Location	Company
99-357-08n Permit still open report not yet submitted	MS113	date pending date pending date pending date pending	0.5 0.3 0.3 8.9	Huxley, IA; Arlington, Wi; Fowler, IN; Santa Isabel, PR	Mycogen
99-274-10n	MS095		No plantings	Huxley, IA; Arlington, Wi; Fowler, IN; Santa Isabel, PR	Mycogen
99-110-05n	MS083	5/14/99 5/4/99 5/12/99 5/25/99 5/3/99	0.57 0.8 0.82 0.62 1.1	Wilkin County, Renville County, Jackson County, Brown County, Winona County, MN	Mycogen
99-078-10n	MS082	5/2/99 5/4/99 5/13/99 4/30/99 5/8/99 5/3/99 5/8/99 5/11/99 5/7/99 5/1/99 5/3/99 5/4/99 5/12/99 4/30/99 4/28/99 5/12/99 5/12/99 5/19/99 5/10/99 5/27/99 5/14/99 4/22/99 5/14/99 4/26/99	3.0 1.5 1.5 0.58 0.76 0.76 1.4 0.84 1.28 2.6 0.75 0.62 1.0 0.7 0.83 0.28 1.6 0.87 0.75 0.01 0.25 2.15 0.62 0.62	Columbia County, WI Jackson County, WI Jefferson County, WI Richland County, ND Cass County, IL Champaign County, IL Scott County, IA LaSalle County, IL Dawson County, NE Story County IA Madison County, IA Pierce County, WI Sac County, IA Plymouth County, IA Calhoun County, MI Decatur County, IN Benton County, IN York County, NE Butler County, NE Clay County, SD Dixon County, NE Santa Isabel, PR Dane County, WI Cass County, ND	Mycogen
98-267-02n	MS059	12/11/98, 2/17/99, 4/1/99, 4/5/99, 4/15/99	5.28	Santa Isabel, PR (total for all plantings is 5.28 acres)	Mycogen
98-127-07n	MS052	6/6/98	0.05, 0.5	Marshalltown, IA Huxley, IA	Mycogen
98-027-02n	MS043	2/26/98, 3/13/98, 7/3/98, 9/18/98 10/9/98 10/27/98	1.7 1.9 2.5 1.2 0.44 0.51	Santa Isabel, PR	Mycogen
97-178-02n	MS032a	7/27/97	0.25	Del Mar, DE	Mycogen
97-059-04n	MS028	4/15/97	2.0	Huxley, IA	Mycogen
97-059-02n	MS026	7/1/97	3.0	Santa Isabel, PR	Mycogen

11/16/01

Dow AgroSciences LLC
5501 Oberlin Drive
San Diego, CA 92121

00-136-018

Dow AgroSciences

USDA Notification #	Tracking #	Planting Dates	Acreage	Field Trial Location	Company
98-040-10N	CRN-US-TX-98-49	5/6/98	0.07	Hale County, TX	Pioneer
98-040-12N	CRN-US-HI-98-52	6/18/98	0.45	Kekaha, HI	Pioneer
98-040-13N	CRN-US-PR-98-75	6/1/98	0.12	Salinas, PR	Pioneer
98-072-20N 98-128-19N	CRN-US-IA-98-42 CRN-US-IA-98-	5/4/98, 5/19/98	1.08,	Polk County	Pioneer
98-155-01N	CRN-US-HI-98-191	7/8/98, 7/27/98	0.38, 0.20	Kekaha, HI	Pioneer
98-296-03n	CRN-US-HI-98-270	3/16/98, 4/16/98, 6/17/98	0.009, 0.001, 0.001	Kekaha, HI	Pioneer
99-028-01R	CRN-US-CP-99-007	3/31/99 – 4/30/00	1.65 0.08 0.62 0.71 0.67 0.03 0.60 0.06 0.01 0.01 0.06 0.01 0.03 0.23 0.01 0.01 0.01 0.02 0.01	Kauai County, HI Kossuth County, IA Linn County, IA Polk County, IA Bureau County, IL Champaign County, IL McDonough County, IL Tipton County, IN Gratiot County, MI Kandiyohi County, MN Saline County, MO Cass County, ND York County, NE Salinas, PR Beadle County, SD Obion County, TN Hale County, TX Eau Claire, WI Rock County, WI	Pioneer

1/16/01

Volume 1

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 1	Microbial B.t. Cry1F (truncated) Delta-Endotoxin: Maize-Insect-Pest Susceptibility Study	Herman, R.A. and Korjagin, V.A.	22

Volume 2

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 2	Characterization of Inserted Genes in Cry1F Maize Line 1507	Hondred, D.	17

Volume 3

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 3	Phosphinothricin acetyltransferase (PAT) protein: In Vitro Digestibility Study	Glatt, C.M.	17

Volume 4

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 4	Characterization of Expressed Cry1F Protein in Maize Tissues (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial Expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures	Young, D.L. and Herman, R.A.	71

Volume 5

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 5	Non-target Exposure and Risk Assessment for Environmental Dispersal of Cry1F Maize Pollen	Wolt, J.D.	244

Volume 6

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 6	Environmental Fate of Cry1F Protein Incorporated into Soil	Halliday, W.R.	87

Volume 7

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 7	Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate	Hoxter, K., Porch, J., Krueger, H.	32

Volume 8

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 8	Chronic Exposure of Folsomia Candida to Bacterially Expressed Cry1F Protein	Halliday, W.R.	122

Volume 9

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 9	B.t. Cry1F Delta-Endotoxin: A 48-Hour Static-Renewal Acute Toxicity Test with the Cladoceran (Daphnia magna) Using Bacterially Expressed B.t .Cry1F Delta-Endotoxin, and Pollen from Maize Expressing B.t. Cry1F Delta-Endotoxin	Drottar, K.R. and Krueger, H.O.	21

Volume 10

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 10	Evaluation of the Dietary Effect(s) on Honeybee Development Using Bacterially Expressed B.t. Cry1F Delta-Endotoxin and Pollen from Maize Expressing B.t. Cry1F Delta Endotoxin	Maggi, V.L.	53

Volume 11

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 11	Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: A Dietary Toxicity Study with Green Lacewing Larvae	Hoxter, K., Porch, J., Krueger, H.	28

Volume 12

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 12	Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: A Dietary Toxicity Study with the Ladybird Beetle	Hoxter, K., Porch, J., Krueger, H.	32

Volume 13

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 13	Cry1F Bacillus thuringiensis var. aizawai Delta Endotoxin: A Dietary Toxicity Study with Parasitic Hymenoptera	Hoxter, K., Porch, J., Krueger, H.	33

Volume 14

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 14	Waiver Request: Fish Toxicity Text With Transgenic Maize (Corn) Containing Bacillus thuringiensis var. aizaqoi (B.t.) Cry1F Delta-Endotoxin	Mayes, M.	18

Volume 15

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 15	Transgenic Corn Expressing Bacillus thuringiensis var. aizawai (B.t.) Cry1f Delta Endotoxin: A Dietary Toxicity Study with the Northern Bobwhite	Gallagher, S., Grimes, J., Beavers, J.	38

Volume 16

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 16	Field Survey of Beneficial Arthropods Associated with Bacillus thuringiensis Cry1F Maize	Higgins, L.	20

Volume 17

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 17	Efficacy of Cry1F Events TC1360 and TC1507	Moellenbeck, D. and Peters, M.	89

Volume 18

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 18	Cry1F Binding Studies	Masson, L., Adang, M. and Schwab, G.	38

Volume 19

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 19	Resistance Management Plan for Transgenic Maize Expressing the Cry1F Insecticidal Protein from Bacillus thuringiensis var. aizawai	Bystrak, P. and Moellenbeck, D.	20

Volume 20

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 20	Supplement to MRID 44714801: Supplemental Data - Product Characterization Data for Bacillus thuringiensis var. aizawai Cry1F Insect Control Protein as Expressed in Maize	Shanahan, D.	78

Volume 21

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 21	Supplement to MRID 44691101: Supplemental Data for Acute Oral Toxicity Study in Mice: Cry1F Bacillus thuringiensis var. aizawai delta-endotoxin	Shanahan, D.	17

Volume 22

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 22	Acute Oral Toxicity Study in Mice	Kuhn, J.	11

Volume 23

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 23	Comparison of Amino Acid Sequence Similarity of Cry1F and PAT Proteins to Known allergen Proteins	Meyer, T.	24

Volume 24

CBI Deleted

Volume No.	Title	Author	No. of Pages
Volume 24	Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterization of Text Substances for Biochemical and Toxicological Studies	Evans, S.	298